BIOACTIVE VERSUS IMMUNOREACTIVE ADRENOCORTICOTROPHIN IN HUMAN BLOOD

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PROEFSCHRIFT

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aan allen die mij na staan

CONTENTS

Chapter I GENERAL INTRODUCTION AND SCOPE OF THIS THESIS 1

I.1 The structure of ACTH 2
I.2 ACTH-related peptides 3
I.3 History of the bioassay of ACTH 5
I.4 The International Standard for ACTH 7

Chapter II BIOASSAY OF ACTH 11

II.1 Introduction 11
II.2 Materials and Methods 13
II.3 Results and Discussion 16
II.3.1 Measurement of corticosterone 16
II.3.2 Optimization of the cell suspension 18
II.3.2.1 The effect of shaking 19
II.3.2.2 The effect of calcium 19
II.3.2.3 The purification of the cells 20
II.3.2.4 The effect of pre-incubation 22
II.3.2.5 The elimination of ACTH-inactivating substances by the purification and pre-incubation processes 24
II.3.2.6 The effect of the incubation time 26
II.3.2.7 The effect of hypophysectomy on the sensitivity 28
II.3.2.8 ACTH-like substances and other interfering agents in several batches of bovine serum albumin 29
II.3.3 Characteristics of the bioassay 30
II.3.3.1 Reproducibility 30
II.3.3.2 Specificity 31
II.3.3.2.1 Activity of other hormones 31
II.3.3.2.2 Activity of ACTH-derived peptides 32
II.3.3.3 Sensitivity and precision 38
II.3.4 Comparison of the bioassay with other isolated rat adrenal cell assays 39

Chapter III RADIOIMMUNOASSAY OF ACTH 44

III.1 Introduction 44
III.2 Materials and Methods 44
III.3 Results and Discussion 47
III.3.1 Radiolabelling of ACTH 47
III.3.2 Conditions of incubation and pre-incubation 49
III.3.3 Separation of free and antibody-bound ACTH 49
III.3.4 Evaluation of the radioimmunoassay 51
III.3.4.1 Reproducibility 51
III.3.4.2 Specificity 51
III.3.4.3 Sensitivity and precision 53

Chapter IV BIOASSAY VERSUS RADIOIMMUNOASSAY OF ACTH IN HUMAN BLOOD 56

IV.1 Introduction 56
IV.2 Materials and Methods 57
IV.3 Results and Discussion 59
IV.3.1 The effectiveness of the extraction of ACTH from human plasma using Vycor, and the influence of the plasma extracts on the standard curves 59
IV.3.2 Further validation of the assays for measurement of human plasma ACTH 66
IV.3.2.1 Reproducibility 66
IV.3.2.2 Specificity 67
IV.3.2.3 Sensitivity 68
IV.3.3 Choice of standard 69
IV.3.4 Normal plasma ACTH levels at 900 h, radioimmunoassay versus bioassay 73

Chapter V BIOACTIVE VERSUS IMMUNOREACTIVE ACTH IN PLASMA: CLINICAL STUDIES 78

V.1 Introduction 78
V.2 Materials and Methods 78
V.3 Results and Discussion 81
V.3.1 Basal plasma levels of Immunoreactive ACTH, Bioactive ACTH and cortisol at 900 h 81

V.3.1.1 I-ACTH levels 81
V.3.1.2 B-ACTH levels 83
V.3.1.3 The ratio of I-ACTH to B-ACTH 86
V.3.1.4 Cortisol levels 88
V.3.1.5 Relations between I-ACTH and B-ACTH levels in plasma 89
V.3.1.6 Relations between plasma I-ACTH or B-ACTH and plasma cortisol 91
V.3.2 Circadian rhythmicity of plasma Immunoreactive ACTH, Bioactive ACTH and cortisol 93
V.3.2.1 Control subjects 93
V.3.2.2 Patients with Cushing's disease 95
V.3.2.3 Patients with Addison's disease 96
V.3.3 Circadian variation of plasma Immunoreactive ACTH, Bioactive ACTH and cortisol between 900 h and 1600 h 97
V.3.3.1 I-ACTH 98
V.3.3.2 B-ACTH 99
V.3.3.3 Cortisol 101
V.3.3.4 Comparison with literature 102
V.3.4 Short-term variation in plasma Immunoreactive ACTH, Bioactive ACTH and cortisol between 900 h and 930 h 103
V.3.4.1 Short-term variation in plasma I-ACTH levels 103
V.3.4.2 Short-term variation in plasma B-ACTH levels 104
V.3.4.3 Short-term variation in plasma cortisol levels 105
V.3.4.4 Comparison of the short-term variation between 900 h and 930 h and the circadian variations between 900 h and 1600 h of hormone levels 105
V.3.5 Dexamethasone suppression test 107
V.3.5.1 I-ACTH 107
V.3.5.2 B-ACTH 108
V.3.5.3 Cortisol 109

Chapter VI I-ACTH AND B-ACTH RESPONSE TO INSULIN-INDUCED HYPOGLYCAEMIA 111

VI.1 Introduction 111
VI.2 Materials and Methods 111
VI.3 Results and Discussion 112

SUMMARY 118

SAMENVATTING 124

LITERATURE 131

LIST OF FIGURES 146

LIST OF TABLES 148

NAWOORD 149

CURRICULUM VITAE 150

GENERAL INTRODUCTION AND SCOPE OF THIS THESIS

Plasma ACTH levels belong to the lowest values formed for circulating hormones in man. In the last decade numerous sensitive radioimmunoassays for ACTH have been described as for various other hormones. It would obviously be desirable that hormone levels as determined by their immunoreactivity equal bioassayable levels. However close parallelism between such levels would only be expected when the antiserum used recognizes only the biologically active portion of the ACTH molecule which resides in the NH_2-terminal ACTH 1-10 sequence i.e. its first 10 amino acids. From scarce literature data it appears that differences have been observed between immunoassayable and bioassayable ACTH levels as has also been documented for other hormones (calcitonin: Roos et al 1978; prolactin: Leung et al 1978; LH: Marana et al 1979; PTH: Segre et al 1972). These differences could, certainly in part, be ascribed to the fact that commercially available ACTH antisera have their immunologic determinant in the central portion of the ACTH molecule. This is in concert with the possibility that ACTH-like peptides circulate which while being partially or totally biologically inactive may remain immunoreactivity.

This thesis explores discrepancies between the steroidogenic and immunologic plasma ACTH-activity, a dichotomy which could compromise the clinical usefulness of the radioimmunoassay. First, an adrenal cell preparation was developed with a markedly improved sensitivity enabling the measurement of an amount of 0.85 pg synthetic $hACTH^{1-39}$ (chapter II). Subsequently a radioimmunoassay is described with the use of a commercially available and widely used antiserum (chapter III). A number of different ACTH moieties were analysed for their responses in both assays. After a detailed description of the critical step of extracting ACTH from plasma and validation of the reliability of both types of assay, radioimmunoassayable (I-ACTH) and bioassayable (B-ACTH) ACTH levels measured in the plasma of healthy subjects were compared and the observed differences discussed (chapter IV). Next B-ACTH and I-ACTH levels under physiological and pathological conditions are presented with emphasis on diurnal variation of these levels (V). Finally, the results are given obtained in dynamic studies using insulin-induced hypoglycaemia as a stimulus to increase endogenous ACTH levels (chapter VI).

In this introduction first the structure of ACTH will be surveyed with respect to its bioactivity (section I.1), secondly literature data on ACTH-related peptides will be discussed (section I.2), thirdly the choice of the bioassay which was developed is discussed in the context of historical notes about several ACTH bioassays (section I.3), and finally, for reason of comparison with older bioassays in which the activity of ACTH is expressed in International Units, a short survey is presented about the assessment of the International Standards (section I.4).

I.1 The structure of ACTH

ACTH is a linear polypeptide containing 39 amino acids and with a molecular weight of about 4500. In a number of mammalian species the primary structure of ACTH was elucidated; for porcine by Bell (1954), for ovine and bovine by Li et al (1954;1958), and for human ACTH by Lee et al (1961). Later the sequence was revised for human ACTH by Riniker et al (1972) and for ovine and bovine ACTH by Li (1972) and Jöhl et al (1974). Recently, Pankov et al (1976) and Kawauchi (1978) elucidated the structure of whale ACTH, whereas Drouin & Goodman (1980) predicted the sequence of rat ACTH by studying rat genomic DNA. Table I.1 shows that for all these species the structure of the ACTH molecule is identical for the sequence 1 to 24 and 34 to 39 with minor species differences in the 25 to 33 sequence.

Table I.1

ACTH sequence of mammalian species

	1 20
Sequence common to all known mammalian adrenocorticotrophins	NH_2-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-Pro-Val-
	21 25 33 39
	Lys-Val-Tyr-Pro- Ala-Phe-Pro-Leu-Glu-Phe-COOH
Human, Whale	Asn-Gly-Ala-Glu-Asp-Glu-Ser-Ala-Glu-
Porcine	Asn-Gly-Ala-Glu-Asp-Glu-Leu-Ala-Glu-
Ovine, Bovine	Asn-Gly-Ala-Glu-Asp-Glu-Ser-Ala-Gln-
Rat	Asn-Val-Ala-Glu-Asn-Glu-Ser-Ala-Glu-

The complete amino acid sequence of porcine ACTH was for the first time synthesized by Schwyzer & Sieber (1963). In addition in the same laboratory (Schwyzer & Kappeler 1963) the 1-24 derivative was synthesized. This part of ACTH, common to all mammalian species as far as is known, has full biological

activity and its use is preferred in clinical medicine because it rarely causes adverse, allergic reactions sometimes seen after the use of the complete molecule. After the correction by Riniker et al (1972) the complete revised human ACTH was synthesized by Sieber et al (1972).

The conformation of ACTH is illustrated in fig I.1 - modified folllowing Schwyzer (1977). Helical structures are formed near the NH_2-terminal end and near the COOH-terminus. Between these two regions the amino acid chain could give rise to a β-turn (Lõw et al 1975) as is illustrated by the sequence 20-28.

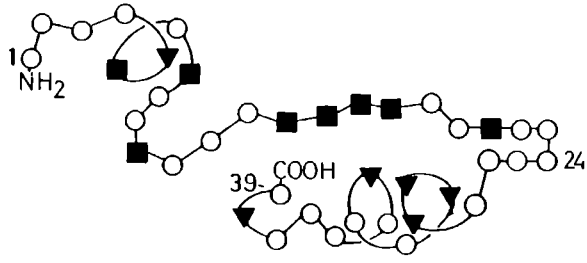

fig I.1 *The structure of ACTH. The positions of the basic amino acids are given by means of the black squares, the acid amino acids by black triangles.*

Furthermore it can be observed from the figure that basic amino acids are localized at the NH_2-terminal part of the molecule. Following the current concept the basic amino acid sequence 15-18 plays a role in the binding and affinity to the receptor (Hofmann et al 1970), whereas the bioactive center of ACTH concerning steroidogenicity in the adrenal cortex is localized in the 5-10 sequence (Schwyzer et al 1971). At the COOH-terminal part of the molecule the acid amino acids predominate. As is known from the work of Imura et al (1967) this part of the molecule is of interest in the protection of ACTH against degradation in human plasma in vitro (see also section II.3.2.5). In section II.3.3.2.2 a number of ACTH fragments were investigated to better define the structure-function relationship.

I.2 ACTH-related peptides

Besides the existence of the 39 amino acid polypeptide, studies both in human plasma and in extracts of pituitary glands or tumors (Yalow & Berson

1971; 1973) indicated the presence of a large molecular weight form, "big" ACTH, which was virtually devoid of biological activity (Gewirtz et al 1974). In addition Orth & Nicholson (1977) found an intermediate form of ACTH with an apparent molecular weight of about 8000. It was suggested that these larger molecules took part as precursors in the synthesis of ACTH itself.
To eliminate the possible aggregation and non-covalent binding of ACTH to serum proteins, Mains & Eipper (1975; Eipper & Mains 1976) examined ACTH activity in mouse pituitaries and an ACTH secreting mouse pituitary cell line. Also they found different molecular weight forms; 31K, 23K, 13K ans 4.5K, the last also known as sequence 1-39, corresponding roughly to the heavier weight forms found in the human: 37K, 24K, 18K and 4.5K (Sueoka et al 1980).

With respect to their origin it is noteworthy that ACTH and the intermediate lobe melanophore stimulating hormones (α-and β-MSH) are closely related because both contain the amino acid sequence $ACTH^{4-10}$, the heptapeptide core. α-MSH shares amino acid sequence 1-13 of ACTH acetylated at the NH_2-terminal end leaving $ACTH^{18-39}$ (CLIP). β-MSH, a portion of β-LPH, shares only the heptapeptide core of $ACTH^{4-10}$ and is in the human only an artificial extraction product (Scott & Lowry 1974). The recently discovered opioid peptide - β-endorphin - corresponds to the COOH-terminal part of β-LPH (Li & Chung 1976).

From the work of Mains et al (1977) and Nakanishi et al (1977) evidence has been obtained that ACTH and β-LPH are derived from a common precursor protein. As ACTH (4.5K) and β-LPH (12K) together account for only half the molecular weight of the precursor molecule, the remaining part (16K) of the precursor had to be elucidated. Nakanishi et al (1979) utilizing a recombinant DNA technique, determined the primary structure of the bovine precursor molecule based on the determination of the nucleotide sequence of cDNA. As is shown in fig I.2 this precursor molecule contains at its COOH-terminal portion β-LPH separated from ACTH by a cleavage site lys-arg. In the cryptic 16K region a sequence almost similar to the sequence $ACTH^{4-9}$ in α-MSH and β-MSH was found. Therefore, a part of the 16K fragment is named γ-MSH. γ-MSH immunoreactivity was detected in the human pituitary by radioimmunoassay (Tanaka et al (1980). It contains an identical amino acid composition as bovine γ-MSH (Benjannet et al 1980). Recently, Pedersen & Brownie (1980) found a potentiation of the ACTH-induced steroidogenesis by the 16K fragment as was also found by Al-Dujaili et al (1981). This synergism could be mimicked by synthetic γ-MSH peptides (Pedersen et al 1980). Furthermore, Bertagna et al

(1980), using an antibody directed against mouse 16K fragment, found 16K immunoreactivity in plasma of patients with Nelson's syndrome and Addison's disease.

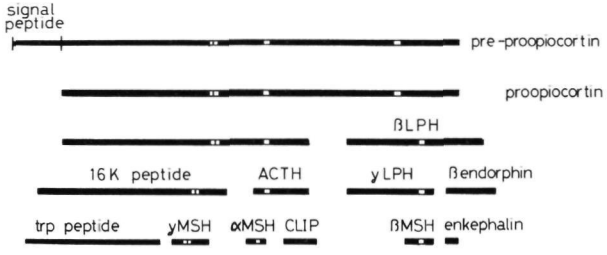

Fig I.2 *Composition of the "ACTH family". It has to be noted that in the human not all of these peptides are synthesized due to a lack of a clear cut intermediate lobe. The open parts indicate the similarity in the amino acid sequence.*

This immunoreactivity was highly correlated with immunoreactive ACTH suggesting a concomitant secretion of the 16K fragment immunoreactivity and ACTH. This finding gives additional information about a possible, coordinate release of peptides derived from the common precursor, because concomitant secretion of ACTH and β-LPH in the human (Gilkes et al 1975; Krieger et al 1977, Tanaka et al 1978) and in the rat of ACTH and β-endorphin (Guillemin et al 1977) was already demonstrated.

Thus a large family of ACTH-related peptides might under certain conditions occur in peripheral blood. Especially under circumstances of a stimulated release of ACTH one has to take into account the possibility of interferences in the assessment of the pituitary-adrenal gland axis function.

I.3 History of the bioassay of ACTH

The classical observation of Smith (1926, 1927, 1930) that hypophysectomy of rats is followed by atrophy of the adrenal cortex and that transplantation of anterior pituitary tissue can restore the normal histology of the adrenal cortex led to the development of ACTH assays. Collip et al (1933) compared the adrenal weight in hypophysectomized rats before and after stimulation with anterior pituitary extracts. Moon (1937) proposed a definition of an unit of

adrenocorticotropic activity using adrenal weight as parameter in normal rats, whereas Simpson et al (1943), Sayers et al (1943) and Li et al (1957) optimized this technique using hypophysectomized rats (table I.2).

The finding of Sayers et al (1946) that ACTH caused a lowering of the levels of cholesterol and ascorbic acid lead to the adrenal ascorbic acid depletion assay (Sayers et al 1948), which detects about 200 µU ACTH and proved to be valuable for many years. However, as the relation of ascorbic acid depletion to corticosteroid secretion is still not well understood, it was desirable to use a more specific parameter of ACTH action on the adrenal cortex, i.e. the steroid release. Nelson & Hume (1955) developed an assay which actually used corticosteroid secretion from the adrenal gland into the adrenal vein in hypophysectomized dogs as a parameter for ACTH activity. This assay permits detection of 1 mU ACTH. Using a simplified technique for the estimation of circulating corticosteroids in the hypophysectomized rat, Guillemin et al (1958) measured peripheral blood corticosteroids after i.v. injection of ACTH which enabled detection of 0.1 mU. Lipscomb & Nelson (1962) perfected the ACTH assay by cannulation of the left adrenal vein, enabling collection of the adrenal venous blood for measuring the released corticosteroids and developed a retrograde technique, i.e. injection of ACTH in retrogade way against the adrenal vein outflow. With this technique the detection of even 5 µU ACTH was possible. However, this technique is very cumbersome. To avoid this Vernikos-Danellis et al (1966) measured the steroid contents of adrenals after injection of ACTH enabling measuring of 25 µU.

The first convenient in vitro bioassay for ACTH was described by Saffran & Schally (1955). The assay was based on the stimulation of the production of corticosteroids by isolated adrenal quarters and enabled detection of 3 mU ACTH. This method was modified by Cohen & Furth (1959) by using transplantable adrenocortical tumor slices (sensitivity 0.25 mU). The development of a method for preparation of isolated rat adrenal cells by Kloppenborg et al (1968) gave a new impulse to the in vitro bioassay. Sayers et al (1971a) improved this method permitting detection of 0.1 µU. Lefkowitz et al (1970a) utilizing purified receptors, described a radioreceptor assay with a sensitivity comparable to that in radioimmunoassays. The most cumbersome but also the most sensitive bioassay reported as far was developed by Chayen et al (1972) using a cytochemical assay in which ACTH causes a loss of reducing potency especially in the cells of the zona reticularis. This technique is related to the loss of ascorbic acid as measured in the original Sayers bioassay. The limit of detection in this assay has been reported to be as low as 0.0005 µU.

Table I.2

Historical survey of bioassays of ACTH

Authors		Method of analysis	Standard	λ	Sensitivity
Collip et al	1933	adrenal weight of hypox rats			
Moon	1937	adrenal weight of intact rats	own standard		± 5 mg
Simpson et al	1943	adrenal weight of hypox rats	own standard		0.02 mg
Sayers et al	1943	adrenal weight of hypox rats	own standard		5 µg
Sayers et al	1948	ascorb.acid deplet. hypox rats	La- 1- A	0.176	0.15 µg
Nelson & Hume	1955	adr. vein 17-OHCS hypox dog	2nd Intern St	0.118	1 mU
Saffran & Schally	1955	corticost. rat adr.slices	2nd Intern St	0.152	3 mU
Li et al	1957	adrenal weight of hypox rats	own standard	0.26	6 µg
Guillemin et al	1958	corticost. trunk blood hypox rats	2nd Intern St		0.1 mU
Cohen & Furth	1958	corticost. mice adr.tumor slices	2nd Intern St	0.167	0.25 mU
Lipscomb & Nelson	1962	corticost. adr. vein hypox rats	2nd Intern St	0.298	5 µU
Espiner et al	1963	cortisol dxm treated sheep			10 µU
Vernikos-Danellis et al	1966	adr. corticost.content hypox rats	ACTHAR (35 U/mg)	0.191	25 µU
Kloppenborg et al	1968	corticost. isol.rat adr. cells	2nd Intern St		40 µU
Lefkowitz et al	1970	radioreceptor assay mice adr.	hACTH (140 U/mg)		1 µU
Sayers et al	1971	corticost. isol. rat adr. cells	3rd Intern St		0.1 nU
Chayen et al	1972	cytochemical assay guinea pig	3rd Intern St	0.09	0.5 nU

In the present study a bioassay had to be developed for measurement of ACTH in human plasma. Three techniques with a great sensitivity are in this respect available: the radioreceptor assay, the cytochemical bioassay, and the isolated adrenal cell assay. The radioreceptor assay depends on attachment of ACTH to receptors present in adrenal cell membranes. This method is technically difficult and may overestimate the true biologically active ACTH value due to binding by biologically inactive ACTH-like peptides. The cytochemical bioassay is extremely sensitive, but it is technically difficult, only a few samples can be processed in one run, and it requires an expensive microdensitometer. Furthermore, it is noteworthy, that with this redox method it is not steroidogenicity that is measured but loss of reducing potency in the reticularis cells of the adrenal cortex (Loveridge et al 1975). For these reasons we employed the isolated adrenal cell assay and made it sensitive enough for measuring plasma ACTH.

I.4 The International Standard for ACTH

For determination of ACTH in plasma samples responses should be compared

with those of a reference preparation. In order to validate the results of different methods in different laboratories International Standards have been established. In 1950 the First International Standard for Adrenocorticotrophin was established by the WHO Expert Committee on Biological Standardization. The material used for this standard was a relatively crude pig ACTH preparation (La-1-A) purified by glacial acetic acid-acetone extraction and prepared in 1946 by the Armour Laboratories. The International Unit was defined as the biological activity contained in 1 mg of the International Standard. After its establishment knowledge about the chemistry of ACTH increased markedly. This led to the establishment of a new standard crude pig corticotrophin, prepared by an extraction procedure developed by Astwood et al (1951) but without an oxycellulose purification. This Second International Standard was assayed against the First International Standard and was assigned a potency of 1.14 IU/mg, thus the International Unit was redefined as 0.88 IU/mg (Mussett & Perry 1956). As assays were used a thymus involution test (Bruce et al 1952) and the ascorbic acid depletion test of Sayers et al (1948) after injection the preparation by the intravenous (i.v.) or subcutaneous (s.c.) route. The i.v.:s.c. potency ratio obtained by the adrenal ascorbic acid depletion assay was 1:1 by definition. However, when an ACTH preparation is assayed by the subcutaneous technique, the potency may be quite different from the potency obtained after intravenous injection. The ratio of potencies obtained by the i.v. and s.c. methods is about 1:3. Because most of the ACTH used in clinical treatment is given subcutaneously or intramuscularly to the patients, a batch of porcine pituitaries, made by Armour Laboratories and purified by glacial acetic extraction, ethyl ether preparation, oxycellulose adsorption and elution, was assayed for the determination of the potency of the Third International Standard by different methods, but potency was established only in the "subcutaneous assay" (Bangham et al 1962). Each ampoule of the Third International Standard contains about 50 µg freeze-dried porcine ACTH together with 5 mg lactose and corresponds to a quantity of 5 IU. Although the Third International Standard is defined exclusively by the s.c. assay, the intravenous route is the most widely used for bioassays and the concentration of ACTH in the blood can also be expressed in i.v. Units. It is of interest, therefore, to note that each ampoule of the Third International Standard contains about 1.5 i.v. units. Therefore, 1 s.c. IU = 10 µg and 1 i.v. IU = 33 µg. As the contents of ampoules of the Third International Standards are dilutions of one master ampoule, dilutions of other equivalent master ampoules were made

in the same way, and are called the Third International Working Standard (IWS).

BIOASSAY OF ACTH

II.1 Introduction

The first authors who practised isolation of adrenal cells for the preparation of cell suspensions were Kloppenborg et al (1968) and Halkerston & Feinstein (1968). Isolating intact cells from their environment was already known for a long time: disaggregation of vertebrate tissue to obtain suspensions of free cells was reported in 1916 by Rous & Jones. Using trypsin they tested several tissues and obtained crops of viable cells. Evans & Earle (1947) isolated cells and brought them in culture. A number of other authors had done similar studies with several tissues (Moscona & Moscona 1952; Essner et al 1954; Younger 1954).

Cultures of adrenal tissues were reportedly obtained from adrenal tumor tissue (Buonassisi et al 1962; Sato et al 1965) and from chopped foetal adrenal glands (Kahri 1966; Milner & Villee 1970). Foetal and also newborn adrenocortical cells lack a number of enzyme activities with which adult adrenocortical cells are endowed (Villee et al 1961). Armato & Nussdorfer (1972) cultivated adult adrenocortical cells. Electronmicroscopy and autoradiography revealed that ACTH could still stimulate adrenal DNA, RNA and protein synthesis in these cells. Cell strains cultivated in vitro for long periods can vary in their morphology (Evans et al 1952) and even undergo spontaneous malignant transformation (Moore et al 1956). Hornsby & Gill (1978) cultured many generations of cells and demonstrated a progressive loss of ACTH-responsiveness in adult bovine adrenocortical cells. In order to obtain reliable information on the behaviour of normal cells we preferred to use freshly isolated cells for the determination of ACTH.

As is stated by Rinaldini (1958): "the preparation of undamaged cells from different tissues in sufficient quantities for chemical estimations must be based on an appraisal of 1) the nature and structure of the intercellular materials to be disaggregated, 2) the action of the agents used for this purpose, and 3) the response of the cells under the particular conditions employed". As is well known the rat adrenal cortex has three distinguishable concentric zones - a thin, outer zona glomerulosa adjacent to the capsule

which surrounds the gland; a thick middle layer, the zona fasciculata; and a moderately thick, inner zona reticularis contiguous with the central lying adrenal medulla. As the cells of the zona fasciculata are thought to respond best to ACTH as far as the secretion of corticosteroids is concerned, special attention must be drawn to the histological appearance of this zona. The fasciculata cells are arranged in long cords disposed radially with respect to the medulla and in tight contact with each other. Among the adrenocortical cell cords intercellular spaces are present, which contain collagen fibers in addition to the ground substance, continuous with the pericapillary space (Kurosumi & Fujita 1975). Therefore isolation of these cells can be achieved by attacking the collagen fibers and/or the ground substance. Usually the enzymes trypsin or collagenase are employed for isolation of adrenal cells. The ground substance is believed to be attacked by the former. Use of trypsin has the advantage that its activity can be stopped at any time by specific inhibitors. The enzyme collagenase is available in purified and in crude form. The latter contains both proteinases and peptidases (Debellis et al 1954) and thus attacks the ground substances as well as collagen fibers. Therefore, disruption of the cells has been effectuated in the present study by means of crude collagenase. However, one should realize that the great variety in quality of enzyme preparations could yet cause disturbances. As is known from the work of Kono (1969), for instance, neither adipose tissue nor cardiac muscle could be dispersed by either trypsin or collagenase alone but only by a mixture of both enzymes. These findings illustrate that preparation of isolated cells is often a matter of trial and error. In most studies concerning isolated rat adrenal cells the digestive enzyme is added to quartered adrenals. In the present study, however, the adrenals were cut into small pieces before enzyme addition in order to give the enzyme optimal contact with the substrate. Besides the use of biochemical agents most of the techniques for the isolation of cells of the adrenal gland employ mechanical agents in tissue disaggregation.

In the light of the foregoing remarks it is obvious that preparations of isolated cells may vary in yield of cells, sensitivity to ACTH, quantity of damaged cells etc. In our study we have tried to optimize the quality of the adrenocortical cell suspension. Bennett et al (1974) demonstrated that sensitivity to ACTH increased after prior purification of cells.
In order to make the cells highly sensitive to ACTH we modified their method and evaluated the usefulness of pre-incubation of the suspension.

In this chapter an isolated rat adrenal cell assay is described with a

sensitivity high enough to measure plasma ACTH levels. The cells - disrupted by means of crude collagenase - were purified by passing through a 5% BSA solution at unit gravity sedimentation. To achieve an additional gain in sensitivity a pre-incubation step was introduced to decrease further enzyme activity which attacks the ACTH molecule. At the hand of testing different ACTH peptides the consequences of the improvements are evaluated.

II.2 Materials and Methods

Materials

ACTH-related peptides

Synthetic hACTH$^{1-39}$ (revised sequence, Sieber et al, 1972, 188 IU/mg as estimated by Schenkel-Hulliger et al, 1974), ACTH$^{1-24}$, ACTH$^{1-16}$, acetyl ACTH$^{1-13}$ NH_2 (α-MSH), hACTH$^{25-39}$, ACTH$^{7-13}$ NH_2 and ACTH$^{11-24}$ were generous gifts from dr W Rittel and dr P Desaulles (CIBA-Geigy Ltd, Basel), hACTH$^{1-32}$ from dr G Fekete (Gedeon Richter, Budapest), ACTH$^{1-10}$, ACTH$^{4-10}$, ACTH$^{11-24}$ and a highly purified human hypophyseal extract (108 IU/mg) from dr W Hondius Boldingh and dr H Greven (Organon, Oss), ACTH$^{1-28}$ and ACTH$^{5-14}$ from dr H Vilhardt (Ferring, Malmö), ACTH$^{11-19}$ NH_2 from dr M Nakamura (Shionogi & Co, Osaka), hACTH$^{18-39}$ (corticotropin-like intermediate lobe peptide, CLIP) from dr H Yajima, ACTH$^{1-4}$ and ACTH$^{5-7}$ from dr K Medzihradszky, ACTH$^{1-23}$ NH_2, ACTH$^{1-17}$ Ala^1, Lys^{17}-4-aminobutylamide and its derivatives Arg^{11}, $Norleu^{11}$ and $Forlys^{11}$ from dr R Geiger (Hoechst, Frankfurt am Main) and β-endorphin from dr J Morley (ICI, Macclesfield).
The standard (synthetic hACTH$^{1-39}$) as well as the ACTH-related peptides were dissolved in ACTH-diluent, lyophilized and stored at 4°C.

Other materials

Vasopressin (270 IU/mg, Sandoz), Angiotensin II (Hypertensin, CIBA-Geigy) LHRH (Hoechst) and TRH (Hoffman-Laroche) were commercially available.
FSH (Roos, 73/519, 8600 U/mg), LH (1st Ref. Prep. of Pit. LH human, 68/40, 664 U/mg), TSH (1st Intern. Ref, Prep. of TSH, 68/38) and Prolactin (1st Intern. Ref. Prep. of Prolactin human, 75/504, 3.25 U/mg) were kindly donated by the MRC (London), corticosterone was obtained from Steraloids, bovine serum albumin (BSA, ORHD 20/21) from Hoechst, nicotinamid adenin dinucleotid phosphate (NADPH) from boehringer, trypan blue from BDH Chemicals Ltd and

collagenase (type I) from Sigma. Dimethyl-dichlorosilane was obtained from BDH Chemicals, methylene chloride from Baker, toluene (p.a.), sulphuric acid (97-99%) and ethanol (p.a.) from Merck. ACTH-diluent was made by dissolving 50 mg BSA in 100 ml 0.9% NaCl after which the pH was adjusted to 3.5 with N HCl. Krebs-Ringer-bicarbonate buffer (KRB) was prepared following Umbreit (1948) and stored at 4°C. Before use 200 mg glucose/100 ml was added to the buffer. This buffer (KRBG) was bubbled through for 10 minutes with a gaseous mixture of 95% O_2 and 5% CO_2. The buffer was supplied with 7.65 mM Ca (KRBGCa) and 0.5% BSA (KRBGACa).

Equipment

Rhesus ampoules were obtained from Vitra Form (St Gallen), polyethylene tubes (17/77 mm) from Sarstedt, polystyrene tubes from Otan (Rijsbergen; the Netherlands), a Biopette from Schwarz-Mann, an incubation apparatus from Lab-line and a spectrophotofluorometer from Aminco-Bowman.

Methods

Siliconization procedure

All glass surfaces were immersed in 5% dimethyl-dichlorosilane in toluene for 6 hours after which the glass was thoroughly rinsed with ethanol and dried by air.

Preparation of crude isolated adrenal cells

For each experiment 5 male Wistar rats (180-200 g) were killed by decapitation. The adrenals were removed, freed of fat, cut in about 10 pieces and transferred to a cold (4°C) 25 ml flask containing a solution of 32 mg crude collagenase and 400 mg BSA in 10 ml KRBG. The tissue was incubated in a metabolic shaker at 100 oscillations/min at 37°C in an atmosphere of 95% O_2 and 5% CO_2. After 50 minutes of incubation the tissue was disrupted by pipetting up and expelling the adrenal tissue with a siliconized Pasteur pipette about 50 times (Haning et al 1970). The large tissue particles were allowed to settle before the suspension was transferred to a cold 100 ml polyethylene tube. Two ml KRBGACa were added to the residue and the material was disrupted again. Both supernatants were combined and centrifuged at 100 g for 10 min (4°C). The supernatant was discarded, the pellet resuspended in 10 ml KRBGACa and the suspension was centrifuged (100 g) again. This washing procedure was repeated once more and after the

final centrifugation the cells were resuspended in 4 ml KRBGACa (non-purified or crude cell suspension) unless otherwise stated.

Preparation of the purified cells

After preparing the crude suspension, as described above, this suspension was purified by layering 1 ml portions upon 8 ml 5% BSA in KRBGCa in 4 different polystyrene tubes as illustrated in fig II.1. After 30 minutes the upper layer was removed by suction and the 5% BSA, which by then contained the purified cells, was diluted regularly to about 110 ml with KRBGCa.

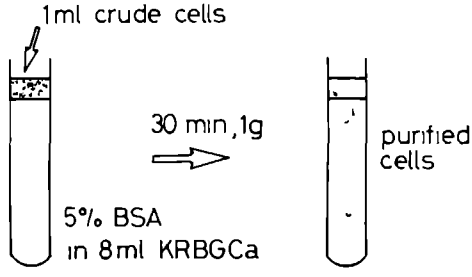

fig II.1 *Purification of adrenal cells*

Incubation procedure

After appropriate dilution of the crude suspension incubation was performed by adding 0.9 ml aliquots to polyethylene tubes which contained 0.1 ml ACTH-diluent. The tubes were placed in a metabolic shaker and the cells were incubated at 37°C under an atmosphere of 95% O_2 and 5% CO_2. When the purified suspension was used the cells were pre-incubated in the tubes for 60 minutes, prior to the addition of ACTH. All observations were made in duplicate except otherwise stated. In a number of experiments the purified cells were not pre-incubated in order to detect the effect of pre-incubation. Incubation time was 120 minutes. In some experiments 0.1 mg NADPH was added to check the quality of the suspension.

Corticosterone measurement

To each incubation tube 6.5 ml methylene chloride was added. After extraction by shaking for 2 min the tubes were centrifuged at 200 g for 10 minutes. The aqueous phase was removed by suction and 1 ml of fluorescence reagent (sulphuric acid: ethanol, 70:30 v/v) was added to 5 ml of the methylene chloride phase. After a contact time of 1 min the organic phase was aspirated and the fluorescence was measured after about 50 minutes using a spectrophotofluorometer at 470 nm (exciting wavelength) and 530 nm (emitting wavelength). Processed corticosterone was used as the standard.

Potency analysis

For each peptide complete log dose-response curves were constructed. The potencies of the peptides were expressed as the relations of the reciprocals of their molar ED50 to the reciprocal of the molar ED50 of $hACTH^{1-39}$. Human $ACTH^{1-39}$ was assigned to the potency of 100 (analogous to Schwyzer et al 1971).

Cell viability

Cell viability was tested by mixing an aliquot of the suspension with an equal volume of trypan blue (100 mg in 100 ml 0.9% NaCl) following Tennant (1964). Cells were counted using a Bürker bright-line haemocytometer. Only those cells were counted which contained lipid droplets and excluded the dye.

Statistical evaluation

In the tables the mean values $\pm$ SD are given. Student's t-test was used to test the statistical significance of differences between groups (P). The correlation coefficients - denoted by r - were calculated following Spearman.

II.3 Results and Discussion

II.3.1 Measurement of corticosterone

It has been shown that the rat adrenal cortex lacks the intrinsic capacity for 17-hydroxylation and is therefore unable to synthesize cortisol or 11-deoxycortisol (Bush 1953). The most important endogenous steroids of the rat adrenal cortex include corticosterone, deoxycorticosterone and aldosterone (Giroud et al 1956; Ward & Birmingham 1960). The fluorometric analysis of corticosteroids, originally developed by Sweat (1954) had been studied extensively by others (Peterson 1957; Guillemin et al 1958, Moncloa et al 1959;

Mattingly 1962, van der Vies 1961). Determination of the specificity, e.g. by Peterson (1957), revealed further that the fluorescence from rat adrenal steroids can be ascribed selectively to corticosterone. Furthermore Sayers et al (1971a) showed that 90% of the fluorescence of steroids obtained from isolated rat adrenal cells was due to corticosterone as was detected before and after purification by thin-layer chromatography.

After mixing with fluorescence reagent there is a steep rise of the fluorescent intensity of corticosterone followed by a plateau.

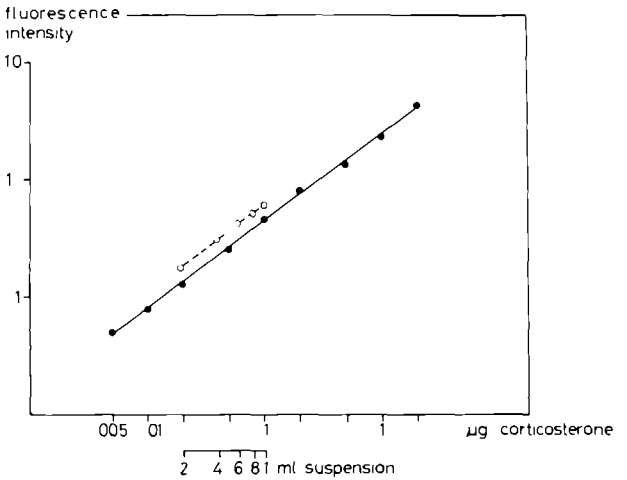

fig II.2 *Calibration curve of corticosterone*

In order to increase the number of estimates which can be performed in one experiment the level of the fluorescence should remain constant for a period long enough to measure a large number of samples conveniently. In this respect two reagents were tested: 70% H_2SO_4/30% ethanol versus 65% H_2SO_4/35% ethanol. The former reagent reached the maximum fluorescence earlier and plateaued for a longer period of time than the latter reagent. For this reason the reagent 70/30 is more convenient and was used as the reagent. The plateau period was also the same for extracts of cell suspension samples to which different ACTH doses were added, as was tested in a separate experiment. About 150 samples can be reliably handled in a period of about 40 minutes.

The fluorescence intensity was linear in the range of 5 ng up to 2000 ng corticosterone (fig II.2). The fluorescence obtained from different volumes

of a pooled cell suspension, incubated with ACTH, parallels the corticosterone standard line. For reasons of convenience all corticosterone estimations were made in rhesus ampoules, whose diameters were previously screened. Only a minor modification of the Aminco Bowman cell holder was needed to render the use of these ampoules. Ten aliquots of a pooled cell suspension, containing 50 pg ACTH/ml were analysed for corticosterone content to estimate the error in the corticosterone measurement; the coefficients of variation revealed 1.27%. The cells were separated from the medium in order to ensure that the corticosterone measurement in adrenal cell suspensions actually means the measurement of corticosterone released from the cells. As appears from fig II.3 the corticosterone content of these cells hardly increased at graded doses of ACTH and equalled the corticosterone content at zero ACTH.

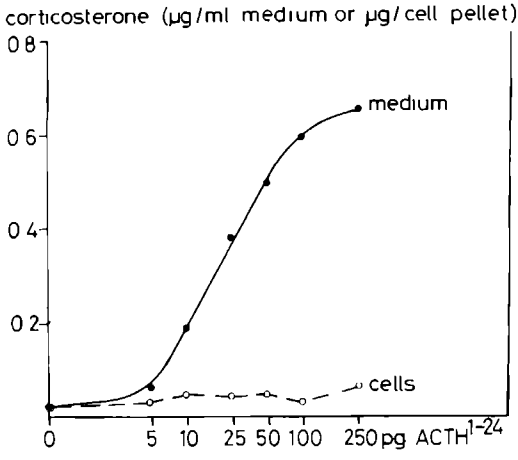

fig II.3 *Measurement of corticosterone in cells and medium. Cells were separated from the medium by centrifugation at 100 g for 10 minutes.*

II.3.2 Optimization of the cell suspension

As stated earlier isolated adrenal cells are much more sensitive to ACTH than adrenal quarters (Kloppenborg et al 1968). The isolated cell system eliminates problems regarding diffusion barriers which are inherent to the classical adrenal quarter preparations. Yet isolated cells present some difficulties. Cells are isolated by means of rather aggressive manipulations such as incubation with crude enzyme preparations and rough mechanical agitation like pipetting. Even after careful washing of the suspension in order to remove collagenase and cell fragments, microscopic examination reveals the presence of a considerable amount of non-viable cells and numerous free cell particles e.g. lipid droplets.

To develop a technique for preparing cell suspensions which are invariably highly sensitive for ACTH as far as the corticosterone production is concerned the following investigations were made:

1. the effect of shaking during the incubation,
2. the facilitating effect of calcium,
3. the influence of purification of the cells,
4. the effect of pre-incubation,
5. the elimination of ACTH-inactivating substances by the purification and pre-incubation processes,
6. the incubation time,
7. the use of adrenals obtained from hypophysectomized rats, and
8. the effect of different BSA batches.

II.3.2.1 The effect of shaking

Cells derived from one suspension were incubated without and with shaking for 100 oscillations / min. Fig II.4 shows only a slight improvement in the response of the cells after shaking. Despite the slight effect of shaking the suspensions were incubated with about 100 opm.

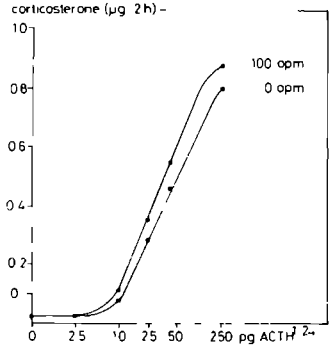

fig II.4 *The effect of shaking upon the ACTH-induced steroidogenesis. Opm: oscillations per minute.*

II.3.2.2 The effect of calcium

The fact that calcium is required for the steroidogenic action of ACTH was first shown by Birmingham et al (1953). It seems to have various effects and its role is not completely clear. Lefkowitz et al (1970b) found a requirement for calcium in the activation of adenylate cyclase by ACTH, Farese (1971) observed a marked effect of calcium on adrenal protein synthesis, which is a prerequisite to stimulate steroidogenesis (Farese & Reddy 1963). Other investigators - working with isolated rat adrenal cells - found a relation between

the ACTH-induced steroidogenesis and the external calcium concentration (Sayers et al 1972; Haksar & Peron 1973; Bowyer & Kitabchi 1974; Perchellet & Sharma 1979; Yanagibashi 1979). Using freshly isolated cells Liotta & Krieger (1977) found the same, but when short-term cultured cells were used, they observed the opposite: with a normal calcium concentration (about 2 mM) the cells were more responsive to ACTH than with a higher (7 mM) concentration. Lowry et al (1973), on the other hand, found no difference using 7.65 mM calcium as compared to the normal concentration. None of these authors observed any influence of calcium per se on the basal secretion of corticosterone. Recently, Neher & Milani (1978) described conditions at which calcium did induce rat adrenal steroidogenesis. Interestingly, Yanagibashi (1979) showed that in this respect a species difference exists. Rat adrenal cells appeared to be insensitive to calcium per se but the basal corticosteroidogenesis in bovine adrenal cells could be stimulated.

We ourselves investigated in a single experiment the effect of calcium in the absence and in the presence of a range of low doses of ACTH. In this experiment at 4 different ACTH doses (1-10 pg) the responsiveness at 7.65 mM calcium was constantly higher than at 2.55 mM, which last concentration is present in KRB (fig II.5). Therefore, 7.65 mM calcium was used during our experiments.

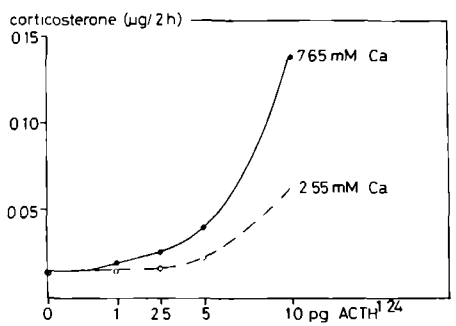

fig II.5 *The effect of 7.65 mM calcium upon the steroidogenesis induced by low doses of ACTH.*

II.3.2.3 The purification of the cells

The presence of non-viable cells and of cell fragments might affect the sensitivity of the assay. First, ACTH may be bound to the receptors of damaged cells, which will not result in corticosteroidogenesis (Halkerston 1968). Secondly, enzymes derived from the broken cells may destroy ACTH and its derivatives (Bennett et al 1974). Therefore we tried to get rid of the cell fragments and broken cells by means of purification of the cells (fig II.1).

The method for purification as described by Bennett et al (1974) - 2 ml of crude suspension layered on top of 20 ml of 2% albumin followed by centrifugation at 10 g for 5 minutes - was not useful in our hands, as just layering the crude suspension (in a 0.5% BSA solution) upon the 2% BSA buffer resulted in an intermingling at the interfase of the two layers. We therefore tried a higher BSA concentration and found that at 5% BSA the separation was optimal. We avoided centrifugation and employed unit gravity sedimentation (Tait et al 1974).

To detect the influence of the time of sedimentation upon the responsiveness of the cells to ACTH, the cells at different ACTH concentrations were incubated for 2 hours, after different sedimentation times. Table II.1 shows

Table II.1

Influence of the sedimentation time on the responsiveness of adrenal cells

	sedimentation time		
$hACTH^{1-39}$ (pg/ml)	30 min	60 min	90 min
0	0.025†	0.030	0.030
5	0.095	0.075	0.055
25	0.300	0.261	0.215
100	0.565	0.500	0.435

†value expressed as µg corticosterone/2 hours incubation

that for sedimentation a time of 30 minutes was appropriate. Periods shorter than 30 minutes were neglected because of the low recoveries of cells. Microscopic examination also showed that after purification only a very small quantity of the cell fragments entered the 5% BSA layer. Recovery of the viable cells after purification for 30 minutes in 10 consecutive experiments varied from 38 to 89% with a mean of 64%.

The quality of the cell suspension can also be assessed by the effect of exogenous NADPH. NADPH is not able to cross adrenal cell membranes and therefore it only stimulates the steroidogenesis of an adrenal homogenate and not that of intact cells (Halkerston et al 1968; Tsang & Stachenko 1968). In our study NADPH was tested and showed a distinct effect upon steroidogenesis in non-purified suspensions, but was hardly effective when the cells were purified (table II.2). This may led to the conclusion that passage through the 5% BSA layer excludes interfering cell debris to a great extent. This is in

Table II.2

Effect of purification and pre-incubation on the ED50 of ACTH and on the NADPH regulated steroidogenesis

	cells non-purified non-pre-incubated		cells purified non-pre-incubated		cells purified pre-incubated	
	ED50 in pg/ml	n	ED50 in pg/ml	n	ED50 in pg/ml	n
$hACTH^{1-39}$ mean ± SD	63.6 ± 21.7	10	31.0 ± 5.6†	7	18.0 ± 6.9††	18
$ACTH^{1-24}$ mean ± SD	20.7 ± 12.2	10	4.6 ± 0.6†	7	2.3 ± 0.8††	11

Netto corticosterone production in ng

	Mean ± SD	n	Mean ± SD	n	Mean ± SD	n
0.1 mg NADPH	316 ± 206	10	29 ± 24†	12	13 ± 12†	18

† significant difference between this value and the value of non-purified non-pre-incubated cells ($P<0.001$).

†† significant difference between this value and the value of non-pre-incubated cells ($P<0.001$).

accordance with the microscopic evidence that only a small quantity of cell fragments enters the BSA medium.

Moreover, table II.2 strikingly shows the improvement on the quality of the suspension by the lowering of the ED50 values by mere sedimentation as the purification procedure. The values were calculated from log dose-response curves obtained in experiments which were performed on separate days.

II.3.2.4 The effect of pre-incubation

As is shown above and as is known from the work of Bennett et al (1974) purification of the adrenal cells removes to a certain degree fragments of cells and enzymes derived from broken cells. However, non-viable cells are still present in the purified suspension. In order to inactivate ACTH-damaging enzymes leaked from these broken cells, a pre-incubation step has been introduced.

Purified cells were pre-incubated during different periods of time and

after addition of 25 pg $hACTH^{1-39}$ a 2 hours' incubation period followed. It appeared that pre-incubation actually influenced the ACTH-induced steroidogenesis; corticosterone production induced by 25 pg $hACTH^{1-39}$ after zero, 30, 60 and 120 minutes' of pre-incubation reaching values of 0.332, 0.382, 0.413 and 0.412 µg respectively. Due to these results it was decided to take 1 hour as pre-incubation time. The enhancement of steroidogenesis as a result of pre-incubation was striking when low doses of ACTH were tested. As fig II.6 shows a remarkable increase in sensitivity was obtained using low doses of either $hACTH^{1-39}$ or $ACTH^{1-24}$ when the purified cells were pre-incubated. The improved sensitivity of the purified cells is also evident from

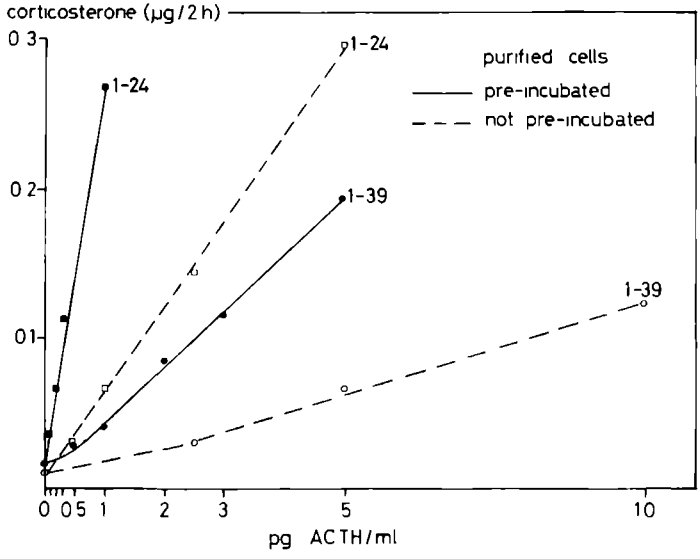

fig II.6 *The effect of pre-incubation for 60 min upon the ACTH-induced steroidogenesis in purified cells.*

the lowering of the ED50 values (table II.2). Then the question arose whether pre-incubation alone - without a purification step - could effectuate the same sensitivity. Therefore the effect of pre-incubation for 1 hour on the ED50 values was studied in three separate experiments. In each of these experiments the sensitivity was enhanced by mere pre-incubation of the cells (ED50: 49.0 $\pm$ 2.6 pg/ml with pre-incubation and 73 $\pm$ 8.4 pg/ml without pre-incubation). When the purified cells were used an even higher sensitivity was reached (ED50: 29.6 $\pm$ 4.0 pg/ml versus 49.0 pg/ml).

II.3.2.5 The elimination of ACTH inactivating substances by the purification and pre-incubation processes.

The theory that the ACTH-attacking substances, present in crude adrenal cell suspensions are inactivated by the combination of purification and pre-incubation, is supported by the results shown in table II.3. Suspension medium - without cells - derived from crude cell suspensions inactivated ACTH whereas such a medium derived from purified, pre-incubated suspensions apparantly did not have such effect.

Table II.3

Effect of a 30 min incubation of the medium of adrenal cells on the steroidogenic activity of $hACTH^{1-39}$

	recovery of ACTH	n
medium of purified,pre-incubated cells†	90.5 ± 28.5%	7
medium of crude cells	29.9 ± 21.8%	7

†Tubes which contained crude or purified,pre-incubated cell suspension were carefully centrifuged and 0.5 ml aliquots of the supernatant (=medium) were transferred to tubes with ACTH. The same had been done with 0.5 ml aliquots of KRBGACa as control. Incubation - without cells - followed for 30 min whereafter the tubes were stored at -20° C until the ACTH content was measured. Recovery of ACTH is expressed as % of the recovery in KRBGACa (=100%).

In order to assess the capacity of the purification and pre-incubation process to remove the ACTH-inactivating cell fragments and substances from the suspension and at the same time to prove that the ACTH-interfering substances could be derived from the debris of cells the following experiment was performed. A concentrated crude cell suspension (7 ml) was divided in two equal parts. To one part 0.5 ml of an adrenal homogenate (2 adrenals homogenized in 0.5 ml buffer) was added and to the other part 0.5 ml buffer. Each suspension was tested for steroidogenic response to both $hACTH^{1-39}$ and $ACTH^{1-24}$, without purification as well as after purification and pre-incubation (fig II.7). The log-dose response curves for $hACTH^{1-39}$ show clearly that the crude suspension to which homogenate has been added is far less sensitive than the non-purified suspension without homogenate. In fact addition of homogenate lowers the ED50 with a factor 2 (fig II.7). After purification and pre-incubation, however, the sensitivities of both the suspension with homogenate added to it and the suspension without homogenate were equal for $hACTH^{1-39}$ as well as for $ACTH^{1-24}$. This means that the purification and

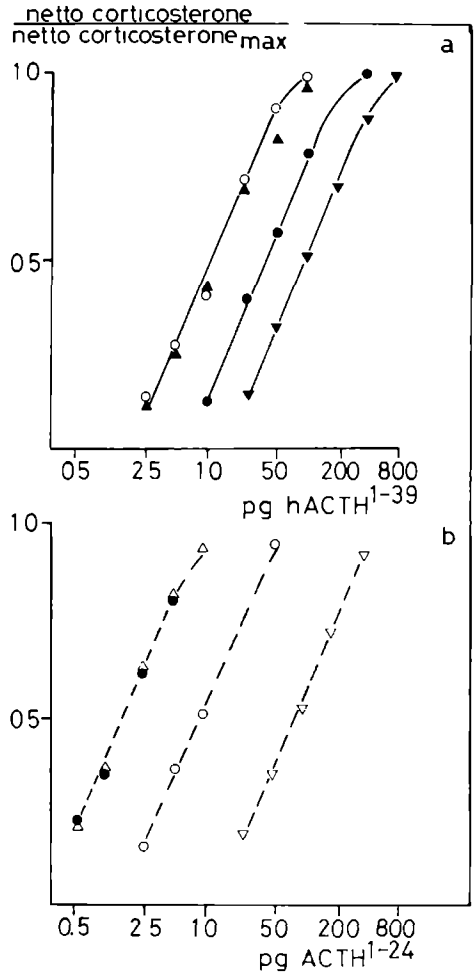

fig II.7 *Effect of cell purification and pre-incubation on the sensitivity to* $hACTH^{1-39}$ *and* $ACTH^{1-24}$ *of adrenal cell suspensions with and without added adrenal homogenate. a: response to* $hACTH^{1-39}$ *of cells in a crude suspension with homogenate addition (▼———▼) and without homogenate (●———●), and in a pre-incubated, purified suspension with homogenate addition before purification (○———○) or without homogenate (▲———▲). b: response to* $ACTH^{1-24}$ *of cells in a crude suspension with homogenate addition (▽— — —▽) and without homogenate (○— — —○), and in a pre-incubated, purified suspension with homogenate addition before purification (●— — —●) or without homogenate (△— — —△). In this experiment the relative potencies of* $ACTH^{1-24}$ *were 520 in the pre-incubated purified suspension, with or without homogenate, 300 in the crude suspension and 76 in the crude suspension with homogenate.*

pre-incubation process is very effective as regards the elimination of the ACTH-inactivating substances. Furthermore, fig II.7 shows that the addition of homogenate lowered the sensitivity for $ACTH^{1-24}$ to a much greater extent than for $hACTH^{1-39}$. The ED50 for $ACTH^{1-24}$ in the pre-incubated, purified suspension was about 8.5 times lower than in the crude suspension whereas for $hACTH^{1-39}$ the ED50 decreased only with a factor 3.5 (table II.2). This difference in relative decrease of the ED50 for both peptides was statistically significant ($P < 0.001$). In other words the increase in sensitivity, which resulted from the purification and pre-incubation procedure was more pronounced for $ACTH^{1-24}$ than for $hACTH^{1-39}$. These results endorse those of Lowry et al (1973) who found $ACTH^{1-24}$ to be 5 to 7 times more potent than the complete hormone. An explanation for these findings was given by Bennett et al (1974) who established that peptide breakdown in isolated adrenal cell suspensions occurred to a less extent for $ACTH^{1-39}$ as compared to $ACTH^{1-24}$. This difference in metabolism decreased by purification of this suspension. In view of these facts it is concluded that addition of adrenal homogenate to the cell suspension (fig II.7) enhances the breakdown for $ACTH^{1-24}$ to a greater extent than for $hACTH^{1-39}$

In order to delineate the cause of the difference in sensitivity change for $ACTH^{1-24}$ and $hACTH^{1-39}$ by the use of pre-incubated, purified cells, we measured the ED50's of $hACTH^{1-32}$ and of a human hypophyseal extract in crude and in pre-incubated, purified cell suspensions. Table II.4 shows that the change in sensitivity for $hACTH^{1-32}$ and for the hypophyseal extract was similar to that for $hACTH^{1-39}$. Therefore it is assumed that the observed difference in potency between $ACTH^{1-24}$ and $hACTH^{1-39}$ cannot be explained by the presence of the COOH-terminal sequence 33-39 in the complete hormone. Imura et al (1967) studied the stability of synthetic ACTH peptides in human plasma in vitro and found that $ACTH^{1-26}$ was more liable to degradation than $ACTH^{1-39}$. These findings together might suggest that the sequence 26-32 is involved in the protection of the hormone.

II.3.2.6 The effect of the incubation time.

After pre-incubation of the purified cells for 1 hour the subsequent exposure of the cells to a maximal dose of ACTH resulted in an increased steroid production already within 2 min. Afterwards an almost linear increase the corticosterone production was found to plateau after about 180 min. As is shown in fig II.8 the corticosterone production increased linearly to about 2 hours with low doses as well.

Table II.4

Relative potencies of different ACTH peptides assayed by corticosterone production

	crude cells	purified pre-incubated cells	n	P
$hACTH^{1-39}$	100	100		
human hyp. extract	50 ± 6	47 ± 7	5	>0.1
$hACTH^{1-32}$	98 ± 7	99 ± 6	5	>0.1
$ACTH^{1-24}$	200 ± 52	528 ± 74	10	<0.001

Several authors, who used high doses of ACTH, reported a lag period before a corticosterone production could be detected. This lag period varies from 3 min (Sayers et al 1971b; Richardson & Schulster 1972) to about 6 min (Kitabchi & Sharma (1971). We found with a dose of 200 pg ACTH an increase in corticosterone production within two minutes already. The rate of steroidogenesis in response to maximal or low doses of ACTH was constant until about 120 minutes. These findings agree with those of others who reported this time to vary between 90 minutes (Sayers et al 1971b) and 240 minutes (Falke et al 1975).

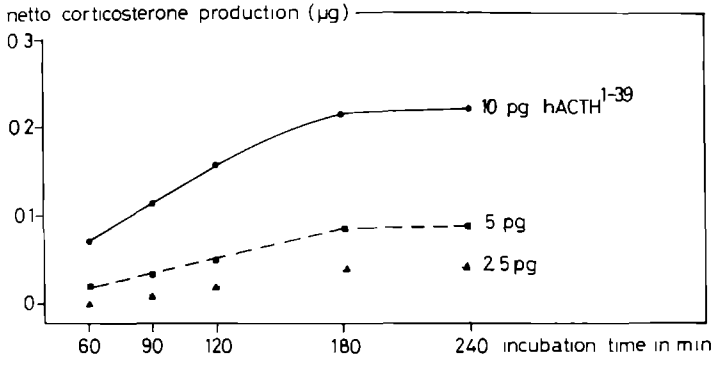

fig II.8 *The effect of the incubation time upon the steroidogenesis induced by low doses of ACTH in pre-incubated, purified cells.*

II.3.2.7 The effect of hypophysectomy on the sensitivity.

Hypophysectomy in rats results in atrophy of the adrenals (Smith 1930) and a diminished secretion of corticosteroids after ACTH injection (Liddle et al 1962). Apparently in contradiction to these reports Sayers & Beall (1973) found that cells isolated from adrenals of rats that had been hypophysectomized 2 days earlier are more sensitive to ACTH than such adrenal cells obtained from normal rats.

These findings led us to investigate this phenomenon in our system. As is shown in table II.5 hypophysectomy of rats - performed by the parapharyngeal approach - two days earlier resulted in a significantly lower ED50 by about a factor 2, when the crude suspensions were compared to those of normal rats The results of our experiments show that nó statistically significant difference in ED50 was observed when pre-incubated, purified cells derived from the same batches of cells were employed. Thus it appeared that in our system - using pre-incubated, purified cells - hypophysectomy per se did not increase sensitivity.

Using the complete hormone Kitabchi et al (1974) found a decrease of the ED50 from 9.0 µU in adrenal cell suspensions of normal rats to 3.2 µU in isolated adrenal cells of rats hypophysectomized 48 hours before. This decrease is comparable to the decrease we found (table II.5) in crude suspensions. Sayers & Beall (1973) used $ACTH^{1-24}$ and detected an ED50 of 3.34 pg for cells of hypophysectomized rats as compared with an ED50 of 27.3 pg for cells from intact rats. This decrease is greater than the decrease found with the complete hormone. As is known $ACTH^{1-24}$ is much more degraded by ACTH-attacking enzymes in isolated adrenal cell suspension than $ACTH^{1-39}$ (Bennett et al 1974; Goverde et al 1980).

Table II.5

Effect of hypophysectomy on the ED50 in crude and purified, pre-incubated adrenal cell suspensions

	ED50, pg $hACTH^{1-39}$/ml			
cell suspension	rats hypophysectomized	rats normal	n	P
crude	38.8 ± 17.5	72.9 ± 17.2	8	<0.01
purified, pre-incubated	13.7 ± 7.0	17.4 ± 4.0	8	>0.1

It is known that corticosteroidogenesis as induced by ACTH is lower after a 2-day hypophysectomy in vivo (Liddle et al 1962) as well as in vitro (Sayers & Beall 1973). This implies a lower metabolic activity and presumably also a lower level of ACTH-degrading enzymes. So, when crude adrenal suspensions from hypophysectomized rats are used, fewer ACTH-attacking enzymes might be present as well. Consequently, this involves a higher sensitivity to ACTH. This hypothesis is supported by the finding that after pre-incubation and purification of cells no further gain in sensitivity could be observed (table II.5)

Moreover, Kitabchi et al (1974) found no significant gain in sensitivity when the second messenger analog dibutyryl cyclic AMP was tested (ED50 0.07 mM in adrenal cell suspensions of normal rats versus 0.06 mM in those of hypophysectomized rats). These data suggest that events before cAMP formation are responsible for the gain in ACTH sensitivity. The finding of Sayers & Beall (1973) is also in agreement with the hypothesis mentioned above. The authors demonstrated that in isolated cells of adrenals of rats hypophysectomized 14 of 28 days earlier ACTH showed neglectable corticosteroidogenesis, although it showed about the same decrease in ED50 for cAMP production as compared with the cells obtained from two-days hypophysectomized rats against the ED50 in cells of normal rats.

II.3.2.8 ACTH-like substances and other interfering agents in several batches of bovine serum albumin.

As is known from the work of Sayers et al (1973) some BSA batches contain a factor which stimulates steroidogenesis in isolated rat adrenal cells. This will result in high control values. By means of glass powder extraction - as described in Chapter IV - we estimated the ACTH content of one batch of BSA (Sigma, fraction V). The value obtained proved to be 1.5 pg bioactive ACTH-like activity/mg BSA. As 5 mg of BSA was generally used in the bioassay, the control value when this batch of BSA was used, contained 7.5 pg of ACTH activity, which obviously causes a loss in sensitivity. The use of such batches should be avoided.

Furthermore,in addition to an ACTH-like activity other substances decreasing the sensitivity of the bioassay may be present as well. Fig II.9 illustrates this decreasing effect of some commercially available BSA batches. One of them had a dramatic effect on the standard curve

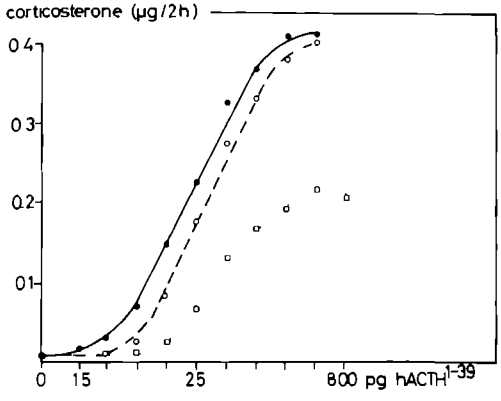

fig II.9 *The effect of different BSA batches on the sensitivity of the standard curve. Concentrated crude cells were divided in three parts. Each portion was purified separately by passage through a 5% BSA layer. Each layer contained BSA derived from different batches. After purification the suspensions were diluted appropriately. Each final dilution proved to contain the same concentration of adrenal cells.*

II.3.3 Characteristics of the bioassay

II.3.3.1 Reproducibility

Cell suspensions were usually diluted evenly to a total volume of about 110 ml. In order to find out whether the adrenal cells are equally distributed over the different tubes an experiment was performed in which the same ACTH dose was added to each 6th tube of a long series of tubes. The results are shown in fig II.10.

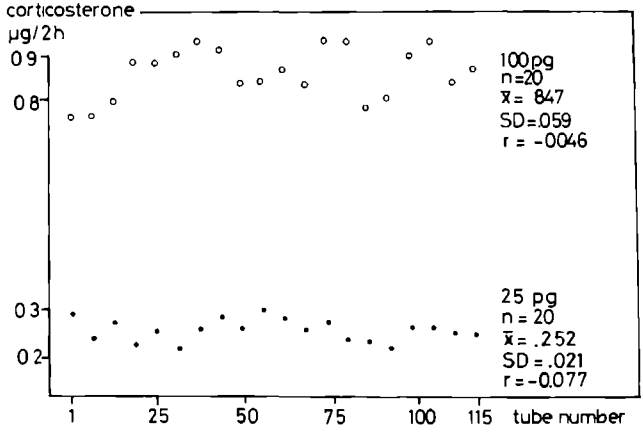

fig II.10 *The reproducibility in the bioassay as measured in two separate experiments for two different doses of hACTH*$^{1-39}$.

The coefficients of variation were 7.0 and 8.3% for the doses 100 pg and 25 pg $hACTH^{1-39}$ respectively.

II.3.3.2 Specificity

II.3.3.2.1 Activity of other hormones

Trophic hormones other than ACTH have been reported to induce corticosteroidogenesis. The data in literature, however, are conflicting. Some authors did find a stimulating effect of FSH and/or LH in vivo (Phillips & Poolsanguan 1978) or in vitro (Vinson et al 1976; Bell et al 1979), whereas others did not (Guillemin et al 1958). The steroidogenic potency of a number of pituitary and hypothalamic hormones as well as of angiotensin II was tested in this study to check the specificity of the assay. It appears from table II.6 that none of the hormones derived from the hypothalamic-pituitary

Table II.6

Specificity of the bioassay

			corticosterone production µg/2 h^{T}
control value			0.010††
$hACTH^{1-39}$	25	pg	0.292
Angiotensin II	0.1	µg	0.009
	1	µg	0.011
	10	µg	0.167
	100	µg	0.510
FSH	0.044	µg	0.010
	0.44	µg	0.008
LH	0.77	IU	0.008
	7.7	IU	0.010
TSH	0.0015	IU	0.008
	0.015	IU	0.014
Prolactin	0.0065	IU	0.015
	0.065	IU	0.013
Vasopressin	0.37	µg	0.012
	3.7	µg	0.008
LHRH	0.25	µg	0.008
	2.5	µg	0.012
TRH	1	µg	0.008
	10	µg	0.007

^{T} values are the mean of duplicate determinations

††corticosterone production of the cells during incubation without ACTH

regıon was able to ınduce a steroıdogenıc response ın the doses used. Besıdes ACTH angıotensın II ıs known to stımulate adrenocortıcal steroıdogenesıs as well, both ın vıvo (Laragh et al 1960; Bıron et al 1961) and ın vıtro (Peytremann et al 1973; Albano et al 1974; Fredlund et al 1975). So, the fındıng that ın our system angıotensın II ın suprapharmacologıcal doses enhanced the cortıcosterone productıon was not unexpected.

Fınally β-endorphın was tested as well. As ıs shown ın fıg II.11 no

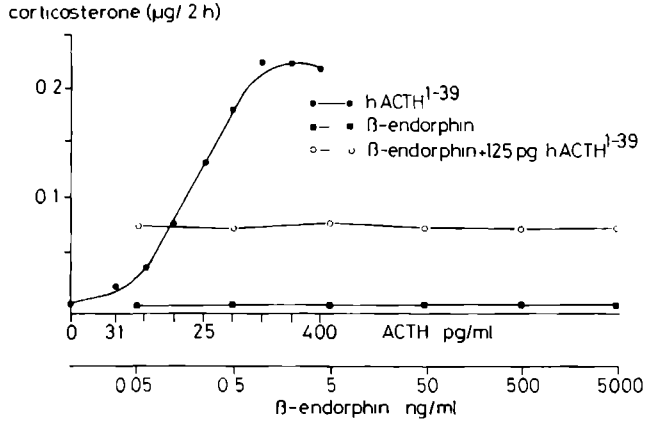

fıg II.11 *The effect of dıfferent doses of* β*-endorphın on the cortıcosteroıdo-genesıs wıthout and wıth hACTH*$^{1-39}$ *(12.5 pg).*

steroıdogenıc actıvıty of β-endorphın was observed up to a concentratıon of 5 µg. Thıs result ıs ın contrast wıth the fındıng of Shanker & Sharma (1979) who observed a clear steroıdogenıc actıvıty wıth an ED50 of 0.1 µg. As ıs shown by Carter et al (1979) β-endorphın potentıates the α-MSH actıvıty. Thıs suggested the possıbılıty that - because α-MSH ıs also actıve ın our system - β-endorphın may potentıate ACTH to a certaın degree. Therefore we tested the effect of β-endorphın ın our system ın the presence of ACTH (12.5 pg). By doses up to 5 µg of β-endorphın the steroıdogenıc actıvıty ınduced by ACTH was not enhanced.

II.3.3.2.2 Actıvıty of ACTH-derıved peptıdes.

The structure-actıvıty relatıonshıp of ACTH-derıved peptıdes was examıned usıng the ısolated adrenal cell system. The actıvıty of hACTH$^{1-39}$ was compared wıth the bıologıcal actıvıtıes of shortened ACTH molecules.

As table II.7 and fig II.12 show shortening of the COOH-terminal part of $ACTH^{1-39}$ to $ACTH^{1-28}$ gave virtually no decrease in steroidogenic activity. A definite increase in potency occurred when $ACTH^{1-39}$ was shortened to $ACTH^{1-24}$ and $ACTH^{1-23}$. By further shortening of the COOH-terminal part the potency decreased dramatically: $ACTH^{1-16}$ had about 1/1000, acetyl $ACTH^{1-13}NH_2$ (α-MSH) about 1/5000 and $ACTH^{1-10}$ about 1/100,000 of the potency of the complete hormone. Loss of three more amino acids, but now at the NH_2-terminal part of the molecule, was responsible for a further decrease of the potency, whereas $ACTH^{5-14}$ showed a greater steroidogenic capacity than $ACTH^{4-10}$. The results showed that there is no difference as far as the maximal steroidogenic production is concerned, when the sequence $ACTH^{4-10}$ was unaltered. This in agreement with the report of Schwyzer et al (1971).

From studies of Hofmann et al (1970) it appeared that the basic amino acids $Lys^{15}Lys^{16}Arg^{17}Arg^{18}$ of the native ACTH molecule are very important for binding of the ACTH molecule to its receptor. This is in agreement with our observation of the very low biological activity of the ACTH moieties that had been deprived of all or some of these amino acids. Note the activities of the 5 ACTH molecules in the right part of figure II.12

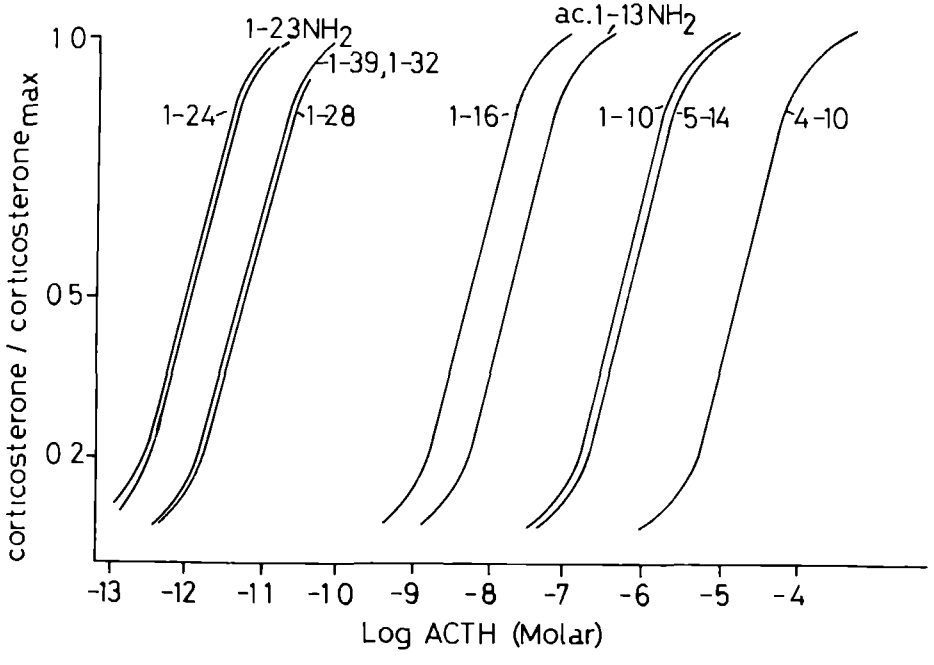

fig II.12 *Log-dose response curves for ACTH peptides which contain the* $ACTH^{5-10}$ *sequence.*

From investigations concerning ACTH conformation Lõw et al (1975) concluded that the acidic terminal part of the native molecule from amino acid 26 onwards may protect the basic core 15-18 against degradation by trypsin-like enzymes. Thus the native hormone might be protected against degradation to a certain extent during its transport in blood from the pituitary gland to its target organs. In our experiments it was found that $ACTH^{1-24}$ is clearly more potent than $ACTH^{1-39}$, a finding in agreement with Lowry et al (1973) but in contrast

Table II.7

Potencies of ACTH-derived peptides

peptide	potency	SD	n	
a) with the sequence 5-10				
$hACTH^{1-39}$	100			
$hACTH^{1-32}$	99	6	5	
$ACTH^{1-28}$	77	12.5	5	
$ACTH^{1-24}$	528	74	10	
$ACTH^{1-23}NH_2$	416	130	5	
$ACTH^{1-16}$	0.089	0.029	7	
acetyl $ACTH^{1-13}NH_2$ (αMSH)	0.022	0.004	6	
$ACTH^{1-10}$	0.00082	0.00011	5	
$ACTH^{4-10}$	0.000021	0.000012	5	
$ACTH^{5-14}$	0.00062	0.00019	4	
b) without the sequence 5-10				highest dose tested
$ACTH^{1-4}$	nd[†]		3	100 µg
$ACTH^{5-7}$	nd		3	100 µg
$ACTH^{7-13}NH_2$	nd		5	100 µg
$ACTH^{11-24}$	0.00032	0.00011	9	
$ACTH^{11-19}NH_2$	0.00067	0.00025	5	
$hACTH^{25-39}$	nd		2	1 µg
$hACTH^{18-39}$	nd		3	1 µg
c) analogs[††] with and without modifications at position 11				
Lys^{11} peptide	32	5	5	
Arg^{11} peptide	36	5	5	
$Norleu^{11}$peptide	0.45	0.16	5	
$ForLys^{11}$peptide	0.46	0.09	5	

[†] nd: not detectable

[††] analog: βAla^{1},Lys^{17} ACTH-(1-17)-4-aminobutylamide

with the findings of Schwyzer et al (1971) and of Liotta & Krieger (1975). Of course it should be realized that our data of the biological potency were obtained in an in vitro system in which the degradation of the ACTH molecule might be obviously different from degradation in vivo. One might speculate that the biological activity is essentially dependent on the availability of the binding sites of the ACTH molecule that reach the receptor molecule in a system in which the enzymatic degradation plays only a minor role. That is how Lõw et al (1975) explained the stronger biological potency of the $ACTH^{1-24}$ molecule as compared with $ACTH^{1-39}$.

Besides the binding site of the ACTH molecule there is another part - the active center - which is involved in the biological activation, residing in the $ACTH^{4-10}$ region (Schwyzer et al 1971).

The steroidogenic potencies of peptides without the sequence $ACTH^{4-10}$ were compared to the activity of the standard $hACTH^{1-39}$. The results are shown in table II.7 and fig II.13. As was expected the COOH-terminal end of ACTH, exemplified by $hACTH^{25-39}$ and $hACTH^{18-39}$ respectively, showed a non-detectable response with doses up to 1 µg.

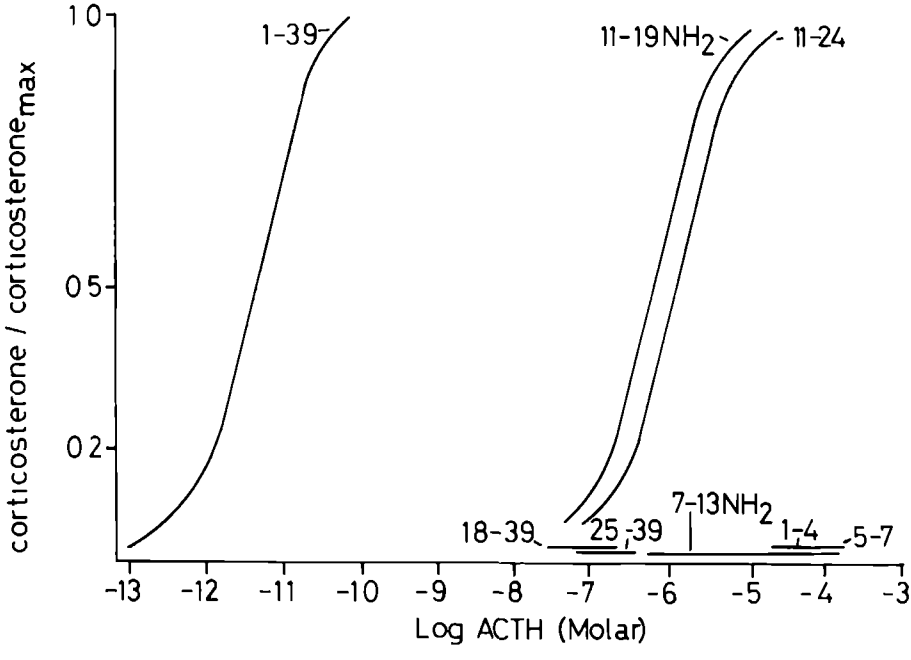

fig II.13 *Log-dose response curves for ACTH peptides without the 5-10 amino acid sequence.*

The peptides $ACTH^{1-4}$, $ACTH^{5-7}$ and $ACTH^{7-13}NH_2$ evoked no steroidogenic response, not even in doses as high as 100 µg.

Our finding of an albeit low but still measurable biological activity of the peptides $ACTH^{11-24}$ and $ACTH^{11-19}NH_2$ is in conflict with other reports. Seelig et al (1971) found that $ACTH^{11-24}$ was able to inhibit the steroidogenic activity of $hACTH^{1-39}$ in pharmacological doses, as was also found by Ramachandran (1974) with $ACTH^{11-19}NH_2$, whereas these peptides did not reveal steroidogenic activity. Remarkably, Nakamura (1972), who used $ACTH^{11-18}NH_2$ observed a potentiating effect of the ACTH-induced steroidogenesis in vitro as well as in vivo, whereas the peptide itself showed no steroidogenicity. In our preparation lower doses up to 50 ng $ACTH^{11-24}$ as well as $ACTH^{11-19}NH_2$ showed neither inhibition nor potentiation of ACTH (fig II.14).

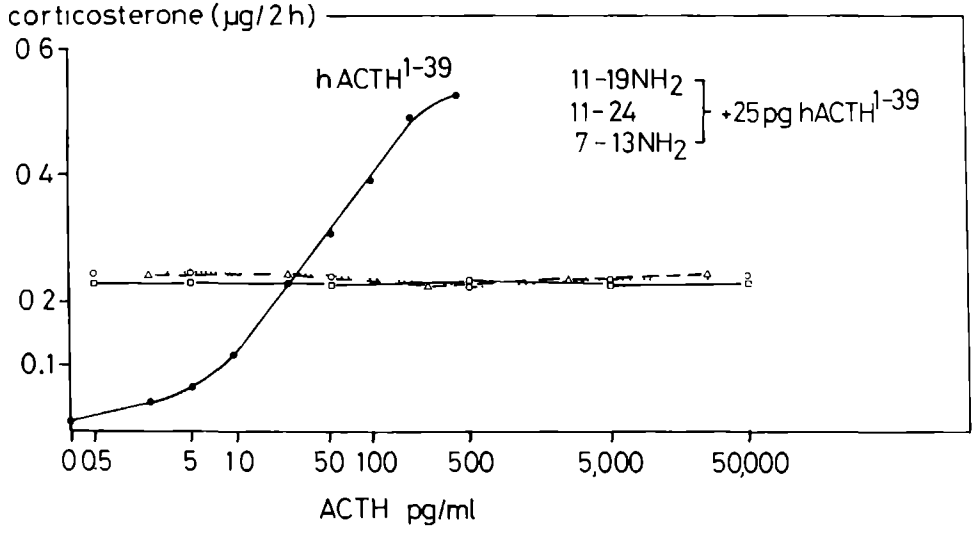

fig II.14 *The effect of different doses of* $ACTH^{11-19}NH_2$ (o- o), $ACTH^{11-24}$ (□——□) *and* $ACTH^{7-13}NH_2$ (Δ— — —Δ) *upon the steroidogenesis induced by 25 pg* $hACTH^{1-39}$.

It is noteworthy that Glossman & Struck (1976) did find formation of cAMP induced by $ACTH^{11-24}$ in a huge dose of 100 µg - in the presence of the GTP analogue $Gpp(NH)_p$. Furthermore, lipolysis in rat adipocytes could also be induced by the $ACTH^{11-24}$ sequence (Opmeer et al 1978) to about the same degree as by $ACTH^{1-10}$. Moreover $ACTH^{11-24}$ contains information for CNS activity (Greven & de Wied 1977). As is known the sequence 15-18 which contains basic amino acids is responsible for the binding and/or the affinity to the

receptor (Hofmann et al 1970) and therefore the sequence 11-14 is of some interest. In this respect it is remarkable that Schwyzer & Eberle (1977) found a second active center in the molecule for α-MSH activity being the sequence 10-13.

In table II.7 and fig II.15 some data are shown about the steroidogenicity of 4 analogs of $ACTH^{1-17}$ with β-ala as the NH_2-terminal end, the amino acid lysine at position 17 and a 4-aminobutylamide at the COOH-terminal position, whereas at the eleventh position lysin was substituted by arginine, norleucine or forlysine. Our data show that arginine did not delete steroidogenicity, whereas the replacement of the basic amino acid by norleucine or by lysine neutralized by formic acid diminished the biological activity by about a factor 80. These observations indicate that it is the positive charge of lysine in position 11 that contributes to the steroidogenic activity of ACTH.

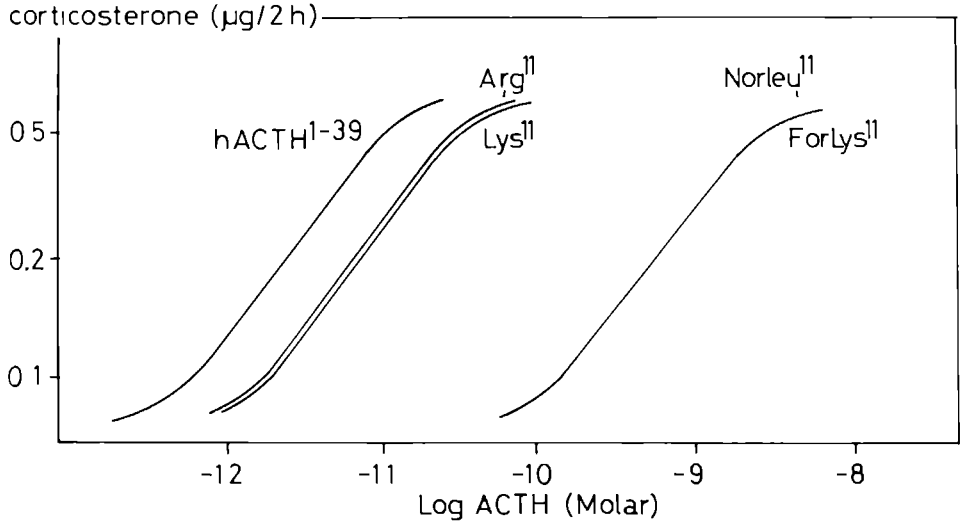

fig II.15 *The effect of the replacement of lysine in position 11 upon the biological activity. The analogs are described in table II.7.*

Our observations are in agreement with the in vivo results of Geiger & Schröder (1973). Similar evidence for a role of the basic amino acid in position 11 was obtained by Li et al (1963). These investigators synthesized a pentadecapeptide $ACTH^{1-10+15-19}$ and found also a diminished biological activity as compared with a peptide without such a deletion.

II.3.3.3 Sensitivity and precision

In order to detect the lowest effective dose of ACTH in the assay, the responsiveness of the adrenal cells was analysed in 10 experiments performed on separate days. In each experiment the corticosterone response to doses of $hACTH^{1-39}$ ranging from 0.5 to 5 pg were measured. Each dose was analysed 5-fold (table II.8). In 5 out of 9 experiments the addition of 0.5 pg ACTH resulted in a significant increase in corticosterone production as compared to the control values. The addition of 1 pg ACTH resulted in a significant increase in corticosterone production in 9 out of 10 experiments. The mean lowest dose which resulted in a significant response was 0.85 ± 0.47 pg $hACTH^{1-39}$/ml.

Table II.8

Effect of low doses of ACTH on corticosterone production (ng/tube) in purified, pre-incubated suspensions

Experiments	Dose $hACTH^{1-39}$ pg/ml (n=5)					
	0	0.5	1	2	3	5
1	27 ± 3		40 ± 5†	68 ± 30	101 ± 10	175 ± 11
2	25 ± 3	51 ± 3	28 ± 3	37 ± 6†	46 ± 3	73 ± 17
3	17 ± 3	29 ± 1†	40 ± 5	86 ± 7	115 ± 9	193 ± 13
4	11 ± 1	11 ± 1	16 ± 1†	29 ± 2	47 ± 4	76 ± 18
5	8 ± 1	14 ± 1†	16 ± 3	34 ± 3	55 ± 5	104 ± 1
6	22 ± 3	26 ± 1†	34 ± 2	58 ± 5	83 ± 2	111 ± 2
7	13 ± 3	19 ± 1†	21 ± 3	40 ± 5	68 ± 7	91 ± 9
8	4 ± 1	5 ± 1	7 ± 1†	9 ± 1	18 ± 2	37 ± 4
9	15 ± 1	20 ± 1†	25 ± 1	45 ± 2	78 ± 2	102 ± 5
10	10 ± 2	11 ± 1	15 ± 1†	28 ± 2	45 ± 3	77 ± 10

†lowest dose at which the value differs significantly ($P<0.01$) from the control value

For $ACTH^{1-24}$ - the doses tested were 0, 0.1, 0.2, 0.3, 0.5 and 1 pg - the lowest limit of detection appeared to be 0.20 ± 0.10 pg/ml.

Precision may be defined as the extent to which a given set of measurements of the same sample agrees with the mean i.e. the amount of variation in the estimation of the hormone (Midgley et al 1969). This error is dependent on the standard deviation of the standard curve and also of the slope of this curve. In 25 experiments performed consecutively for assaying ACTH in human

plasma extracts (chapter IV) over a period of about 1 year, regression lines were calculated for the dose range where the response is linearly related to the log of the dose. In table II.9 are shown:

- the corresponding slopes (b)
- the standard deviation (s), calculated from the formula

$$s = \sqrt{\frac{\Sigma(y - y_{calc})^2}{n - 2}}$$ (Sayers et al 1948)

in which y= the estimated value for a dose, and y_{calc}= the value for a dose as calculated from the equation y= bx + a

- λ= s/b (the index of precision; Gaddum 1953).

The mean values appeared to be for s: 0.0133, for b: 0.2957 and for λ: 0.0464.

Table II.9

Precision of the bioassay

Date	s	b	λ
Aug. 20 1979	0.0093	0.2530	0.0369
Okt. 1	0.0260	0.4936	0.0527
Okt. 4	0.0490	0.6498	0.0755
Okt. 16	0.0247	0.4609	0.0537
Okt. 24	0.0096	0.4023	0.0238
Nov. 8	0.0021	0.2912	0.0730
Jan. 17 1980	0.0072	0.2524	0.0288
Jan. 22	0.0057	0.2887	0.0197
Jan. 24	0.0158	0.2776	0.0571
Febr. 4	0.0098	0.3330	0.0296
Febr. 27	0.0133	0.3661	0.0363
March 7	0.0099	0.2572	0.0385
March 10	0.0060	0.1422	0.0423
March 28	0.0082	0.2876	0.0285
May 19	0.0058	0.1106	0.0524
June 19	0.0048	0.1282	0.0381
June 24	0.0215	0.4954	0.0434
June 26	0.0103	0.2038	0.0505
June 30	0.0066	0.1339	0.0493
July 2	0.0322	0.5497	0.0586
July 8	0.0164	0.3512	0.0467
Aug. 8	0.0137	0.2051	0.0668
Aug. 19	0.0093	0.1811	0.0517
Aug. 20	0.0049	0.1443	0.0339
Aug. 21	0.0098	0.1342	0.0731
Mean	0.0133	0.2957	0.0464
SD	0.0105	0.1463	0.0155

II.3.4 Comparison of the bioassay with other isolated rat adrenal cell assays

Under section II.3.3.2.2 it was already noted that the stimulating action of different ACTH moieties on adrenal corticosterone production differs greatly. The lowest limit of detection for $ACTH^{1-24}$ appeared to be 4 times lower

Table II.10

Historical survey of adrenocortical cell suspensions

Authors	Year	Enzyme	Standard	Sensitivity pg/ml	ED50 pg/ml	λ	Adrenal equivalent[x]	Manipulation
Kloppenborg et al	1968	cls	2nd Intern St	40 μU				
Swallow & Sayers	1969	t	3rd Intern St	300[†]		0.078±0.048		
Haning et al	1970	cls	pACTH	1600[†]	4000[†]		1	
Sayers et al	1971	t	3rd Intern St	3	150[†]	0.009-0.063	0.5	
Kitabchi & Sharma	1971	t	3rd Intern St	15[†]	200		1	
			ACTH (1-24)	5[†]	70			
Nakamura & Tanaka	1971	t	3rd Intern St	150[†]	2000[†]	0.06	1.66	
Richardson & Schulster	1972	cls	3rd Intern St	10	1200		0.33	
Moyle et al	1973	cls	sACTH (173 IU/mg)	100[†]	1750		0.66	
Lowry et al	1973	t	hACTH		50		0.10	
			ACTH (1-24)	1[†]	7	<0.08		
Sayers & Beall	1973	t	ACTH (1-24)	1[†]	3.3±0.34			hypox rats
					27.3±2.2			normal rats
Kolanowski et al	1974	t	ACTH (1-24)	25[†]	380			
Kitabchi et al	1974	t	3rd Intern St		32			hypox rats
Bennett et al	1974	t	ACTH (1-24)	1[†]	5[†]			purified cells
Falke et al	1975	cls	pACTH (75 IU/mg)	10[†]	400[†]		0.2	
Liotta & Krieger	1975	t	synth hACTH (1-39)	2	7-58	0.079		hypox rats
Morita et al	1975	t	3rd Intern St	200[†]	1000[†]			
Mulder	1975	cls	ACTH (1-24)	10-30	100-250	0.057±0.005	0.31	
Finn et al	1976	t	ACTH (1-24)		850[†]			
Porter & Heiman	1977	t	ACTH (1-24)	10			0.27	
Liotta & Krieger	1977	cls/t	3rd Intern St	0.98± 0.10	35	0.05		cultured cells
				12.1 ± 3	240	0.09		normal cells
Braley & Williams	1977	cls	ACTH (1-24)	10	100			
Sala et al	1979	cls	pACTH (150 IU/mg)	4	450		1	
Goverde et al and present study	1980	cls	synth hACTH (1-39)	0.85± 0.47	18.0±6.8	0.046+0.016	0.083	pur.pre-inc. cells
			ACTH (1-24)	0.20± 0.10	2.3±0.8			

[x] adrenal part present in 1 incubation tube

[†] estimated from figure

than for $hACTH^{1-39}$. So, as far as the sensitivity of a method is concerned, the value obtained is dependent on the standard used. The higher potency of $ACTH^{1-24}$ as compared to the complete hormone has been reported by various authors (Schwyzer et al 1971; Lowry et al 1973; Goverde et al 1980). Table II.10 summarizes in chronological sequence data from literature concerning isolated rat adrenal cell assays and includes information about the standard used. It is clear that the first methods had a poor sensitivity compared to the methods that were developed later. The detection limits of the assays increased dramatically, over the years, finally reaching a value as low as 0.85 pg/ml using $hACTH^{1-39}$ and 0.20 pg/ml using $hACTH^{1-24}$. This high sensitivity was reached in this study after purification and pre-incubation of the adrenal cells.

A wide range of values may also be observed when we compare the more clearly defined parameter for sensitivity used by different investigators, the ED50. These values vary from 18 to 4000 pg/ml for the complete hormone and from 2.3 to 850 pg/ml for $ACTH^{1-24}$. In order to obtain a higher sensitivity several modifications had been made: hypophysectomy of the rats; purification of the cells; short-term culture of the cells; purification followed by pre-incubation, and mere dilution of the cells. The following ED50 values for the natural hormone were obtained; after hypophysectomy 32 pg/ml (Kitabchi et al 1974) and 7-58 pg/ml (Liotta & Krieger 1975), after short-term culture of the cells 35 pg/ml (Liotta & Krieger 1977), after pre-incubation of purified cells 18 pg/ml (this study), and mere dilution of the cells 50 pg/ml (Lowry et al 1973). As far as $ACTH^{1-24}$ is concerned (table II.11) it appears that the present method yields the lowest ED50: 2.3 pg/ml using pre-incubated, purified cells as compared with 3.3 pg/ml for the technique with hypophysectomized rats (Sayers & Beall 1973), 5 pg/ml for the technique with purified cells (Bennett et al 1974), and 7 pg/ml for the technique with a diluted assay (Lowry et al 1973).

The reported values of the index of precision (λ) are in close agreement with each other varying from 0.046 (present study) and 0.05 (Liotta & Krieger 1975) to 0.09 (Liotta & Krieger 1977) with cells treated normally. These values are all lower than those reported in bioassays dating from before the development of the isolated adrenal cell system (chapter I).

The term "adrenal equivalent" - indicating the part of one adrenal that is present in one incubation tube - has been employed as parameter for the optimal use of the quantity of tissue. This value ranges from 1.66 (Nakamura

& Tanaka 1871) to 0.083 (present study) indicating a high efficiency of the present method as compared to the adrenal equivalents of other reports (table II.10).

Table II.11

Comparison of isolated adrenal cell assays with $ACTH^{1-24}$ as standard

Authors	Year	Sensitivity	ED50 pg/ml	Manipulation
Kitabchi & Sharma	1971	5† pg/ml	70	
Lowry et al	1973	1†	7	
Sayers & Beall	1973	1†	3.3	hypox rats
			27.3	normal rats
Kolanowski et al	1974	25†	380	
Bennett et al	1974	1†	5†	purified cells
Mulder	1975	10-30	100-250	
Finn et al	1976		850†	
Porter & Heiman	1977	10		
Braley & Williams	1977	10	100	
present study	1981	0.20	2.3	purified cells pre-incubated

†value estimated from figure

RADIOIMMUNOASSAY OF ACTH

III.1 Introduction

The development of a sensitive radioimmunochemical method by Yalow & Berson (1960) has led to the radioimmunoassay for the determination of many hormones including ACTH. As such a method involves a competition between labelled and unlabelled hormones for the binding to antibodies, determination of hormones by means of radioimmunoassay requires the availability of a labelled hormone, a specific antibody and a technique for separating the bound and the free hormones (for review: Berson & Yalow 1973). Of these prerequisites the production of antibodies for ACTH presents one of the main difficulties because ACTH has a low antigenicity, is rapidly degraded in blood, induces the secretion of corticosteroids, which inhibit the immune system, and requires antibodies with a high affinity because of the fact that molar levels of ACTH in human blood are rather low as compared to many other hormone levels.

This chapter describes the method for the radioimmunoassay for ACTH per se.

III.2 Materials and Methods

Materials

Peptides

In the radioimmunoassay the same standard (synthetic $hACTH^{1-39}$, CIBA-Geigy) was used as in the bioassay. Peptides used for crossreactivity studies were described in chapter II. The peptide used for labelling was a highly purified human ACTH hypophyseal extract (Organon, Oss).

Reagents

Bovine serum albumin (BSA, ORHD) was obtained from Hoechst (Frankfurt am Main); sodium phosphate dibasic, potassium phosphate monobasic and sodium acetate (p.a.) from Merck (Darmstadt); diethylbarbituric acid (Barbitone),

diethylbarbituric sodium (Barbitone sodium) and thiomersal from BDH Chemicals (Poole); Quso G32 glass powder used for purification of the tracer from Philadelphia Quartz Co (Philadelphia); charcoal, Norit A, from Fisher Scientific Co (New Jersey); dextran T 70 and Sephadex G 50, fine, from Pharmacia Fine Chemicals AB (Uppsala); lactoperoxidase (3000 IU/mg dry weight), lot 387013, from Calbiochem AG (Luzern); $Na^{125}I$ (11-17 Ci/mg) from the Radiochemical Centre (Amersham); acetone (p.a.), acetic acid (p.a.) and hydrogen peroxide, 30%, from Merck, diluted to 0.003% with distilled water. Standard and unknown samples were diluted in ACTH-diluent, which was made by solving 50 mg BSA in 100 ml 0.9% NaCl after which the pH was adjusted to 3.5 with N HCl. The assay buffer (0.02 M) was prepared by adding 5.742 g sodium phosphate and 0.416 g potassium phosphate to 1 l physiological salt containing 100 mg thiomersal and 0.25% BSA (pH buffer: 7.6). Iodination buffer (0.05 M) was prepared by adding 1.504 g sodium phosphate and 0.108 g potassium phosphate to 100 ml distilled water (pH buffer: 7.0). Veronal buffer (0.07 M) used for separation was made by adding 0.791 g Barbitone, 6.209 g Barbitone sodium, 4.55 g sodium acetate and 100 mg thiomersal to 1 l distilled water (pH. 8.6). As ACTH-free plasma, outdated heparinized blood bank plasma was used. The absence of ACTH in this plasma was tested before use.
The antibody used was purchased from Wellcome (RD 05). It was prepared from a single bleeding of a rabbit immunized with repeated injections of human ACTH in adjuvant. Each bottle contained the lyophilized residue of 0.5 ml of 1:250 diluted antiserum. The antibody was reconstituted in 5 ml of phosphate buffer and stored in aliquots of 0.5 ml at -20°C until use. The antibody was diluted in phosphate buffer to 1:5,000 before addition.

Equipment

Polystyrene tubes, 11 x 40 mm and 16 x 100 mm, were obtained from Otan (Rijsbergen, the Netherlands); a dispensor, adjustable from 0.2 to 0.8 ml, from Oxford (Ireland); an acrylic column (600 x 9 mm) from Pharmacia (Uppsala), a peristaltic pump (R-11) from LKB (Sweden); a centrifuge (Ecco) from Marius Utrecht; a fraction collector (Retriever III) from Isco (Linedu, Nebraska); a gamma counter (NE 1600) from Nuclear Enterprisers. A decantation apparatus as described by Vecsei (1974) was employed but with modifications suitable for gamma ray tubes.

Methods

Radioiodination of ACTH

In this study highly purified human ACTH was used for radioiodination. The procedure is a modification of the method of McIlhinney & Schulster (1974). One µg lactoperoxidase (in 1 µl 0.05 M phosphate buffer) was added to 2.5 µg ACTH (in 15 µl 0.05 M phosphate buffer) followed by an addition of 1 mCi $Na^{125}I$ (in 10 µl NaOH) and 10 µl 0.003% H_2O_2. After a reaction time of 10 sec, 0.5 ml phosphate buffer (0.05 M) was added. After addition of 1.5 ml ACTH-free plasma and subsequent vortexing, ACTH was adsorbed to Quso G 32 according to Berson & Yalow (1968). The mixture was vortexed for 5 minutes and centrifuged at 4°C. The glass sediment was washed with 2 ml aqua dest and centrifuged. ACTH was eluted from Quso by 1 ml of 40% acetone and 1% acetic acid in distilled water during rotation for 30 minutes. After centrifugation the eluate was transferred to another tube and concentrated by a stream of air at room temperature to a volume of about 0.5 ml. After addition of 1.5 ml assay buffer the tracer was stored in fractions of 250 µl at -20°C.

In the assay the tracer was purified by means of gelfiltration immediately before addition. The Sephadex G 50 column (50 x 0.9 cm) was equilibrated with assay buffer, after which about 0.25 ml tracer was transferred to the column. The tracer was eluted with assay buffer and eluate fractions of 2 ml were collected. An appropriate fraction was diluted in assay buffer and added to the assay tubes in 50 µl aliquots. With this purified tracer a 100% binding was performed at antiserum dilution 1·250.

Incubation procedure

One hundred µl of standard or unknown sample were pipetted into each polystyrene tube, after which 50 µl antiserum as well as 300 µl assay buffer were added. Four control tubes, without an antibody, were also set up for the determination of the non-specific binding. The mixture was vortexed and pre-incubated for about 3 days at 4°. After administration of 50 µl of labelled ACTH the assay was accomplished with an incubation for 24 hours at 4°C. The final dilution of the antibody was 1:50,000.

Separation of antibody-bound and free hormones

Activated charcoal (250 mg/10 ml) was suspended in Veronal buffer (0.07 M). After mixing with ACTH-free plasma (1.5/8.5 ml buffer, v/v) 0.2 ml samples of the separation suspension (4°C) were brought into disposable caps, which were placed on the incubation tubes. After that the suspension was mixed with

the contents of the test tubes by inverting and shaking all tubes - numbering up to 145 - simultaneously. Immediately afterwards the tubes were centrifuged at 3000 g for 10 minutes ($4^{o}C$). After the centrifugation step the tubes were placed in a special decantation device and the supernatants were decanted into tubes and counted in a gamma counter. The percentage of radioactivity of the tracer that was added but not adsorbed to charcoal in the four control tubes in which no antibody was present revealed the amount of non-specific binding. Correction for this non-specific binding in other samples was made by subtraction.

III.3 Results and Discussion

III.3.1 Radiolabelling of ACTH

Under the conditions described above the yield of radioiodinated ACTH in the acetic acid eluate of Quso, expressed as the percentage of radioactivity related to the total amount of radioactivity employed, was 23.7 $\pm$ 5.3% (n=13). The tracer was used in the radioimmunoassay for a period of 2 months. During this period a gradual decrease of binding to the antibody was observed.

Without tracer purification by means of gelfiltration, the non-specific binding, which represents to a certain degree the "damage" of the tracer, was often about 10%. To minimize this damage, the tracer was purified by gel chromatography (fig III.1 upper panel); a small peak appeared in the albumin fractions (5-7) whereas the major peak was eluted in fractions 13 to 18 with smaller weight components collected in fractions 19 to 30. Each of those fractions were analysed for the B_o and the non-specific binding. It is shown in fig III.1 (lower panel) that the radioactive material present in the first fractions binds poorly to the antibody. Using this radioactive material the non-specific binding appeared very great. Furthermore, it is clear that the tracer in fractions 13 to 18 showed a great binding with the antibody and a low non-specific binding. Fractions 19 to 30 contained tracer responsible for a high non-specific binding and a B_o inferior to the B_o obtained when tracers from fractions 13 to 18 were used, which indicates a higher content of "damaged" peptides. From these results it was concluded that in fractions 13 to 18 tracer with the lowest damage was present. The non-specific binding as determined in 30 consecutive experiments using fraction 15 appeared to be 2.98 $\pm$ 0.83%.

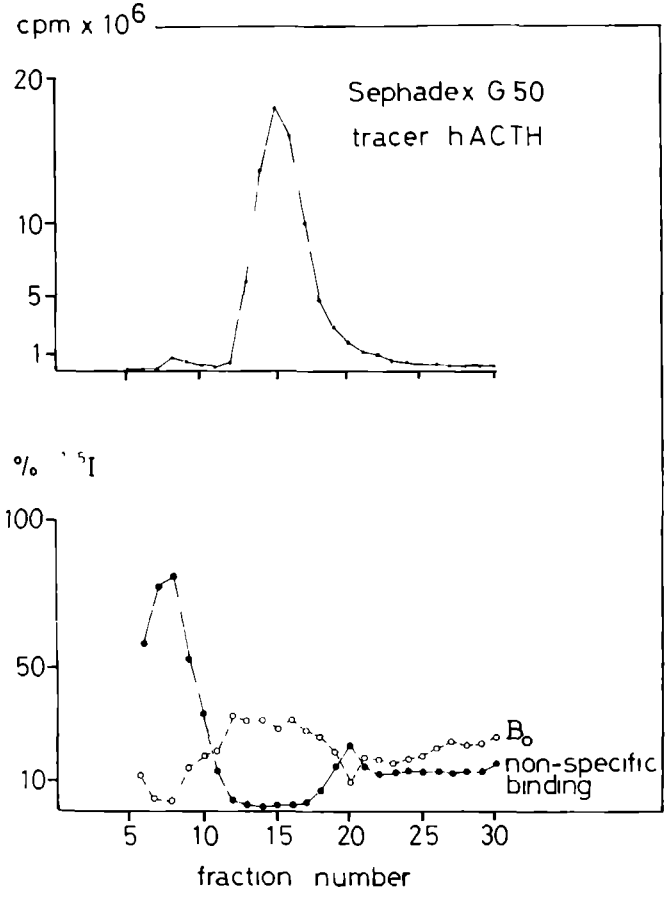

fig III.1 *Chromatographic pattern of the tracer. Upper panel: chromatographic elution pattern of iodinated ACTH following the procedure as described in Materials and Methods. Lower panel:* B_o *and non-specific binding of each fraction. In the assay 5000 cpm radioactivity of each fraction was added to each incubation tube.*

On 8 different occasions the specific activity of the tracer was determined by the self-displacement method described by Chervu & Murty (1975). The activity amounted to 206 ± 39 μCi/μg. As calculated by Galskov (1972) this means an average content of about 0.5 atom ^{125}I per molecule ACTH. Other authors who used lactoperoxidase reported specific activities of 285 ± 30 μCi/μg (McIlhinney & Schulster 1974) and 125 ± 15 μCi/μg (van Dijk 1979). In most studies chloramine T was used as oxidizing agent and the specific

activities obtained showed a great variety in ranges. Landon & Greenwood (1968) reported a wide range of 120 - 690 µCi/µg as did Matsukara et al (1971): 150 - 530 µCi/µg and Galskov (1972): 240 - 800 µCi/µg. Donald (1968) found a smaller range of 500 - 750 µCi/µg which was also reported by Orth et al (1973): 150 - 200 µCi/ µg and by Liotta & Krieger (1975): 260 - 340 µCi/µg. Obviously, the specific activity we found in our study agreed rather with the ranges as reported in literature.

It is of interest that on 2 occasions when the biological activity of the tracer was determined, this activity amounted to 57 $\pm$ 6% of the immunoreactive value. This is in close agreement with the findings of McIlhinney & Schulster (1974) who reported this value to be between 50 and 60%.

III.3.2 Conditions of incubation and pre-incubation

Addition of labelled ACTH can be performed in two ways. Tracer may either be added to the antibody at the same time as unlabelled ACTH, or tracer may be added to the antibody at a later stage than the unlabelled hormone. The effect of the pre-incubation step, which was first described by Hales & Randle (1963) for insulin, was a gain in sensitivity as has been reported earlier by many authors.

III.3.3 Separation of free and antibody-bound ACTH

The last step in the radioimmunoassay is the separation of the free and bound hormones after which both fractions can be quantified by counting the radioactivity. The methods for separation are based upon differences between the characteristics of free and bound hormones. In this respect it is important that free polypeptides can easily adsorb to materials like charcoal (Herbert et al 1965), talc and Quso (Rosselin et al 1966). The use of charcoal coated by plasma as developed for the radioimmunoassay of ACTH by Donald (1968) permits a rapid and easy method.

In order to detect the optimal coating of charcoal by means of plasma in our system, several concentrations of ACTH-free plasma were added to the separating medium (fig III.2). It is evident from this figure that the addition of ACTH-free plasma definitively influenced the B_o. The content of 1.5 ml plasma in 10 ml of the separating suspension caused an almost maximal B_o, whereas the non-specific binding was still acceptable. For these reasons this plasma concentration was employed in the separating suspension. It appears that the presence of plasma (-proteins) in the separation suspension

prevents the adsorption of the ACTH-bound antibody to the charcoal in a very effective way. In this respect it is of interest that Rosselin et al (1966) proposed that adsorption of peptide hormones to silicates has been competitively inhibited by other proteins and plasma. Absence of plasma in the charcoal suspension might result in adsorption of the bound fraction as well (fig III.2).

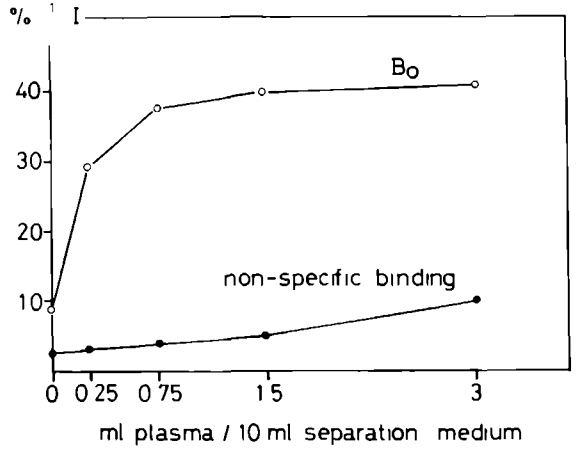

fig III.2 *The effect of the presence of plasma in the separation suspension on the* B_o *and the non-specific binding.*

Fig III.3 shows that the contact time of charcoal influences the percentage of the bound fraction. Therefore the charcoal suspension was added to disposable caps which permits simultaneous addition of the charcoal to all tubes.

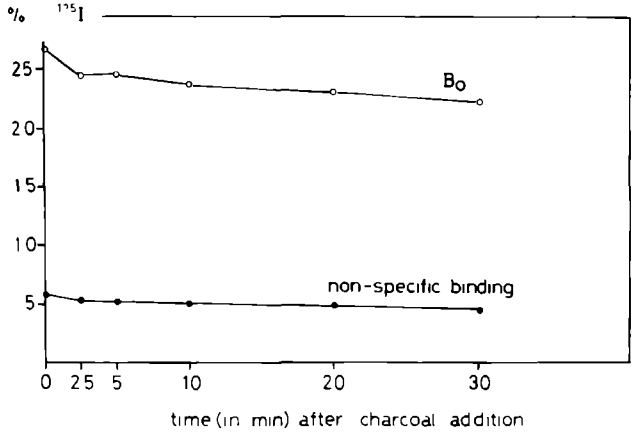

fig III.3 *The effect of the contact time with charcoal on the* B_o *and the non-specific binding.*

Fig III.4 shows that after centrifugation in order to separate the free and bound fractions there was a gradual decrease of the B_o in time. For this reason all bound fractions of one experiment were removed simultaneously. Therefore we adapted the decantation device as developed by Vecsei (1974) for the use of γ-ray counting tubes. In this way a simultaneous decantation of 145 tubes was possible.

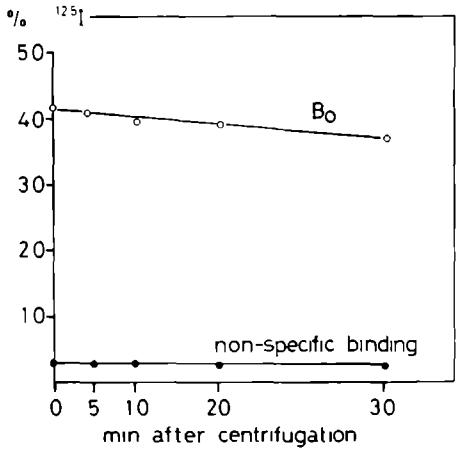

fig III.4 *The effect of time after separation of the bound and free fractions after centrifugation on the* B_o *and the non-specific binding.*

III.3.4 Evaluation of the radioimmunoassay

III.3.4.1 Reproducibility

When 8 standard curves were made in one run, this resulted in a mean standard curve as shown in fig III.5. The coefficients of variation for the doses 0, 10, 25, 50, 100, 200 and 400 pg were 3.3%, 4.1%, 3.0%, 7.4%, 2.8%, 4.1% and 5.5% respectively.

III.3.4.2 Specificity

In order to localize the centre of the ACTH molecule which reacts immunologicaly with the antibody, different ACTH molecules were tested for crossreactivity. In order to study parallelism in reactivity of the molecules the percentages of binding were translated in logits:

$\text{logit} = \ln \frac{(B/B_O)}{(1-B/B_O)}$ (Midgley et al 1969). It is shown in fig III.6 that

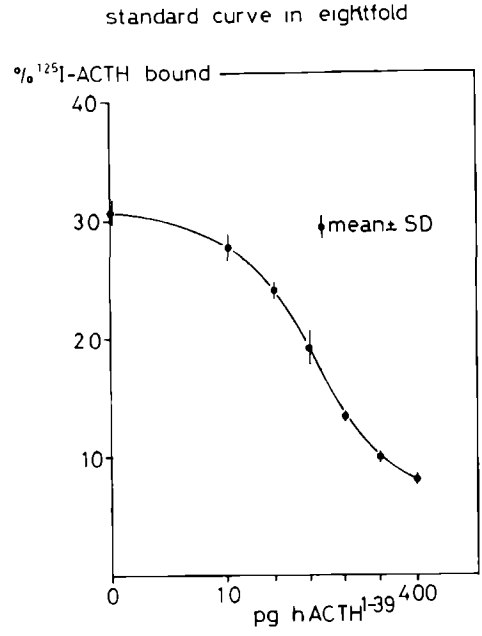

fig III.5 *Standard curve in eightfold.*

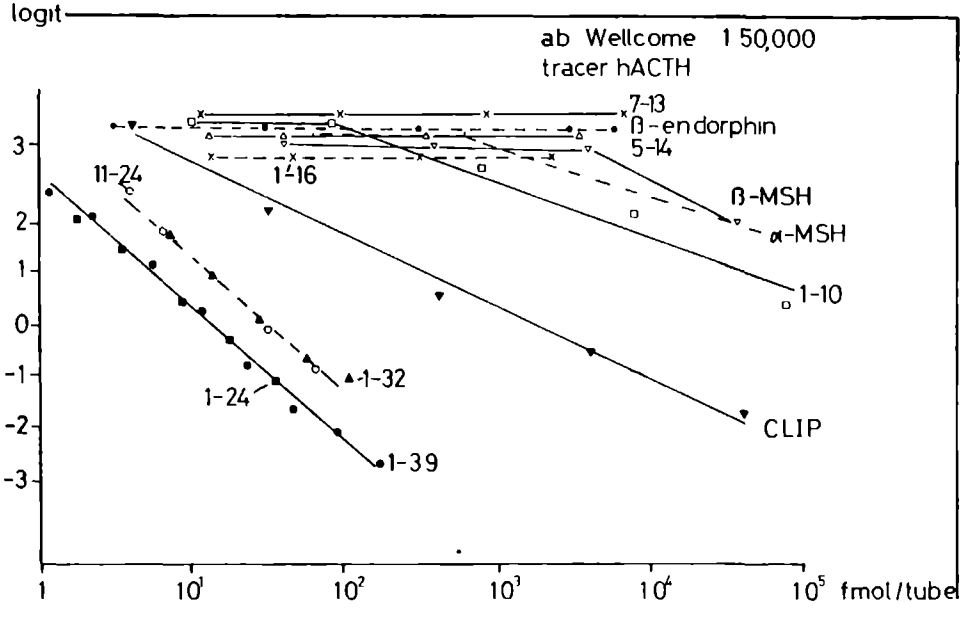

fig III.6 *Crossreactivity of ACTH-derived peptides in the radioimmunoassay.*

on a molar base $ACTH^{1-24}$ equalled the standard $hACTH^{1-39}$. Furthermore, $ACTH^{11-24}$ and $hACTH^{1-32}$ showed a high crossreactivity, whereas other ACTH fragments crossreacted in a negligible way. These findings extend the observation of Croughs et al (1973) and confirms that of Genazzani et al (1974) who found that this antibody binds mainly the amino acid sequence 17-24. This means that in this assay with this antibody peptides can be detected which have a similar sequence of amino acids as $ACTH^{17-24}$. Such peptides may be devoid of a high bioactivity (chapter II) and can lead to a radioimmunological overestimation of ACTH values.

In order to calculate the affinity of ACTH to the antibody (Ka) and to calculate the total number of binding sites in the antiserum (Abo) the data of a standard curve were analysed according to the method of Scatchard (1949), and with corrections as proposed by Chamness & McGuire (1975). As is shown in fig III.7 the Ka was $1.1 \times 10^{11} M^{-1}$, whereas the maximal available binding sites appeared to be 5×10^{-7} mol/l.

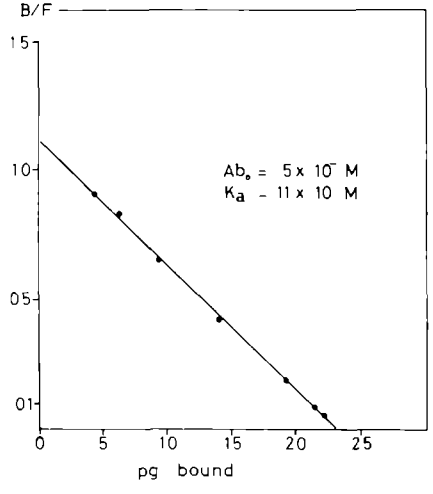

fig III.7 *Scatchard plot of a standard curve.*

III.3.4.3 Sensitivity and precision

The sensitivity of a radioimmunoassay depends on the error in the determination and on the slope of the standard curve (Midgley et al 1969). In order to estimate the percentage of binding (Bm) which is statistically

different from the B_o the following formula was used (Bizollon & Faure 1976): $t = \frac{B_o - B_m}{\sqrt{\sigma^2_{xy}}}$. In this formula: t is the value of Student's t-test which belong to the 95% probability, and σ^2_{xy} the common variance of the standard curve. The B_m was calculated and the corresponding ACTH dose was estimated from the standard curve. In 10 consecutive assays the lowest limit of detection appeared to be 8.8 $\pm$ 2.8 pg $hACTH^{1-39}$/tube.

The mean index of precision - λ (=s/b , Gaddum 1953) - was determined, as described in chapter II, in the dose range where the response is linearly related to the log of the dose. In 10 consecutive assays the mean index of precision appeared to be 0.0278 $\pm$ 0.0081. In literature only very few indices of precision are reported: Voigt et al (1974) found a value of 0.0435 whereas Galskov (1972) found 0.0309.

BIOASSAY VERSUS RADIOIMMUNOASSAY OF ACTH IN HUMAN BLOOD

IV.1 Introduction

Most reported ACTH values in blood of normal subjects collected at about 900 h range from 10 to about 150 pg/ml plasma as measured by radioimmunoassay (Berson & Yalow 1968; Landon & Greenwood 1968; Voigt et al 1974) or by bioassay (Vance et al 1962; Ney et al 1963; Davies 1964; Lefkowitz et al 1970a; Daly et al 1974). Determination of such low values involves the use of very sensitive assays. Moreover many radioimmunoassays as well as bioassays still require a preceding extraction step - with the exception of the radioimmunoassays as described by Berson & Yalow (1968), Galskov (1972), Matsukara et al (1971), Kao et al (1979) and Thoren et al (1981), and the bioassays described by Lefkowitz et al (1970a) and Wolfsen et al (1972) - radioreceptor assay - and Daly et al (1974) - cytochemical assay.

Extraction not only concentrates ACTH but may also avoid the interference with substances which are present in plasma both in bioassay (Fehm et al 1973; Sayers et al 1973; Liotta & Krieger 1975) and in radioimmunoassay (Moldow & Yalow 1980). The earliest but rather time consuming methods for extraction of ACTH from plasma were reported by Sydnor & Sayers (1952) who used the oxycellulose technique and by Williams et al (1961) who used resin adsorption. Recently more convenient procedures have been described. Ferrebee et al found in 1951 that adsorption could occur to laboratory glassware and Stouffer & Lipscomb (1963) observed that ACTH could be eluted from glassware at low pH. These findings led to the development of more simple but still effective extraction procedures (Donald 1967; Ratcliffe & Edwards 1971). A similar procedure was applied in the development of our assays to determine plasma ACTH.

By a direct measurement of ACTH in human plasma disturbances were detected in both our assays. Therefore we applied Vycor glass particles - which gave the best results of the glass powders tested - for the extraction of ACTH from plasma. As the measurement of immunoreactive ACTH (I-ACTH) in the plasma extracts in normal subjects resulted in much higher values than the bioactive ACTH (B-ACTH) values in the same plasma extracts, we scrupulously investigated

the methodology of both assays to establish a reliable determination of immunoreactive and bioactive ACTH in the plasma extracts. In this chapter a reflection of these investigations is presented and the immunoreactive and bioactive ACTH plasma levels in normal subjects are evaluated and discussed.

IV.2 Material and Methods

Materials

Peptides

For the radioimmunoassay as well as for the bioassay synthetic human $ACTH^{1-39}$ was used as the standard. Other ACTH preparations used were: $ACTH^{1-24}$, a human hypophyseal extract (Organon, Oss), the Third International Working Standard (IWS) - obtained from the WHO International laboratory for Biological Standards (Mill Hill, London) - and Cortrophin (purified porcine ACTH, 75 IU/mg, Organon, Oss).

Chemicals

Heparin (5000 U/ml) was obtained from Organon (Oss), acetone (p.a.), acetic acid (p.a.) and hydrochloric acid (37%, p.a.) from Merck (Darmstadt). As extraction glass powder Vycor (code 7930, mesh 325, Corning Glass Works, New York) was employed. A number of other glass particles were tested: Quso G 32 (Philadelphia Quartz Co, Philadelphia), Silicic acid (mesh 100; Mallinckrodt, Frankfurt am Main), Spherosil 200 (Rhône Progil, Lyon) and Florisil (60-100 mesh, BDH). Vycor, silicic acid and Spherosil were previously heated for 48 hours at 700°C and stored at 100°C - as were Quso and Florisil. Trasylol (10,000 IU/ml) was obtained from Bayer (Leverkussen). ACTH-diluent was used as described in Chapter II.

Equipment

Polypropylene tubes (75 x 10 and 52 x 10 mm), polyethylene tubes (100 ml, Sarstedt, Breda), a high speed centrifuge MSE, Hi-spin 21, Crawley, England), and a rotation apparatus (Heidolph) were used.

Methods

Plasma collection

Samples of 25 ml of human blood, obtained from 17 laboratory collaborators - 14 men and 3 women, aged 19 to 43 year - at 900 h in the morning, were drawn from the antecubital vein into heparinized syringes and poured directly into 100 ml ice-cooled plastic tubes containing 50 µl heparin. Plasma was obtained by centrifuging without delay at 4°C, Trasylol was added and the plasma immediately frozen at -20°C and stored until extraction. Plasma samples were centrifuged again immediately before extraction. A pool of outdated blood bank plasma without detectable ACTH activity was used as ACTH-free plasma.

Extraction procedure

The extraction described is a modification of the method of Ratcliffe & Edwards (1971). The whole procedure was performed at 4°C. Vycor glass particles - 25 mg suspended in 0.25 ml distilled water - were added to plasma samples up to 4 ml. After a 10 sec agitation of the glass powder-plasma mixture the tubes were rotated for 30 min and subsequently centrifuged at 5000 g for 10 min. The supernatant was discarded, the pellet resuspended in 2 ml distilled water and the suspension centrifuged again at 5000 g for 10 min. The supernatant was again discarded and the pellet washed by vortexing with 2 ml N HCl. After centrifugation the supernatant was discarded and ACTH eluted from the pellet by rotation for 30 min with 1 ml of acetone: distilled water (50:50, v/v). After centrifugation 0.9 ml aliquots of the supernatants were transferred to other tubes. These supernatants were placed in a water bath at 50°C and evaporated to dryness overnight under a fine stream of air.

Immediately before the assay the dried extract was regularly reconstituted in 0.25 ml of ACTH diluent by vortexing. In order to minimize the presence of ultrafine glass particles in the supernatant the tubes were subsequently centrifuged now at 30,000 g for 60 minutes. In the bioassay as well as in the radioimmunoassay 0.1 ml aliquots were employed.

Construction of standard curves with the use of Vycor extraction

Extracts of unknown plasma samples were calibrated against a processed standard curve in the bioassay as well as in the radioimmunoassay. This standard curve was constructed as follows; to an appropriate volume of ACTH-free plasma (2 or 4 ml samples) a solution of 0.1 ml ACTH-diluent - containing 2000 pg of standard ($hACTH^{1-39}$) - was added and extracted as described. The extract of this standard sample (usually performed in duplicate) was reconstituted in 0.5 ml diluent. After agitation and centrifugation at 30,000 g for 60 minutes,

0.25 ml was transferred to a polystyrene tube containing already 0.25 ml diluent. This tube was agitated and serially diluted through 6 more tubes each containing 0.25 ml diluent. From each dilution 0.1 ml was transferred to assay tubes, obtaining the processed standard curve. In order to control whether an acceptable recovery was obtained non-processed standard curves as described in chapter II and III were constructed as well.

Assays

Details of the bioassay and the radioimmunoassay have been described in chapter II and III respectively.

Statistics

Statistical significance between two means of groups was determined by using Wilcoxon's two sample test (P). Correlation coefficient (r) was calculated using Spearman's rank correlation test.

IV.3 Results and Discussion

IV.3.1 The effectiveness of the extraction of ACTH from human plasma using Vycor, and the influence of the plasma extracts on the standard curves

Using Vycor the recovery of tracer added to ACTH-free plasma appeared to be $\pm$ 75%, which is quite satisfactory when compared to the recoveries obtained with other glass powders (fig IV.1 and table IV.1).

Many ACTH extraction procedures have reportedly been performed at room temperature. ACTH can degrade in plasma (White & Gross 1957; Mirsky et al 1959; Imura et al 1967), which is mainly observed when measuring bioactive ACTH. Using Vycor the effect of room temperature during the extraction procedure has been compared with the procedure at $4^{o}C$. It is shown in table IV.2 that in the bioassay standard ACTH, added to two ml ACTH-free plasma, is recovered to a greater extent at $4^{o}C$ than at room temperature. In a single experiment the recovery as measured in the radioimmunoassay was also higher at $4^{o}C$ than at $20^{o}C$.

The influence of plasma extracts on the standard curves in the radioimmunoassay as well as in the bioassay is shown in fig IV.2. As appears from the figure the Vycor extraction did not influence the radioimmunoassay standard curve. Plasma extracts in which other glass powders (Quso, silicic acid) were used did interfere with the standard curve in the radioimmuno-

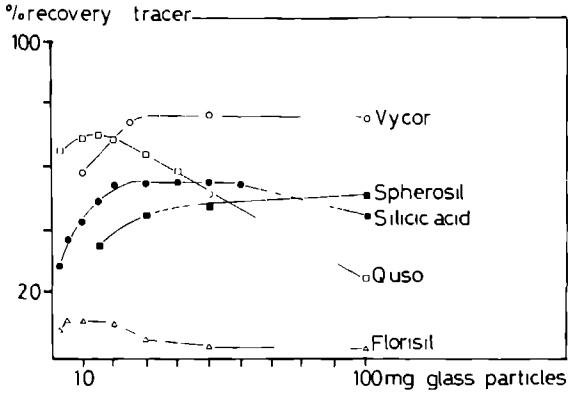

fig IV.1 *The extraction recovery of tracer ACTH, added to 4 ml ACTH-free plasma samples, using different quantities of different types of glass powder. The extraction procedure for Spherosil and silicic acid was the same as described for Vycor. Using Quso and Florisil the procedure was modified as follows: after the water wash step ACTH was eluted with 1 ml acetic acid: acetone: distilled water (1:40:59; v/v); to avoid the presence of an insoluble residu 0.9 ml eluate aliquots were mixed with 1 ml acetone: HCl (40:1; v/v) and 30 min later a centrifugation step followed after which the supernatants were dried as described.*

Table IV.1

Comparison of the effect of different materials and their activation at 700°C upon tracer extraction from 4 ml ACTH-free plasma as performed at 4°C

	adsorption	H_2O wash	HCl wash	sediment	recovery
	% loss	% loss	% loss	% loss	%
60 mg sil.acid[×]	22.6	3.2	19.2	3.9	51.1
60 mg sil.acid (700°C)[+]	21.5	2.6	4.7	4.0	67.2
100 mg Spherosil[×]	39.1	4.4	8.6	2.7	45.2
100 mg Spherosil (700°C)[+]	36.3	3.8	3.3	2.9	53.7
25 mg Vycor[×]	15.0	2.8	16.0	8.1	58.1
25 mg Vycor (700°C)[+]	12.1	2.1	1.4	6.3	78.1

[×]glass not activated; values are the mean of triplicate estimations

[+]glass activated at 700°C before use; values are the mean of triplicate estimations

Table IV.2

The effect of temperature during extraction upon the recovery of $hACTH^{1-39}$ in ACTH-free plasma as measured by bioassay

		% recovery	n
2 ml plasma + 250 pg $hACTH^{1-39}$	(20° C)	59.1 ± 4.9	5
idem	(4° C)	70.2 ± 7.5 †	5

† P <0 01 versus 20°C

assay. The influence of the Vycor extract on the bioassay standard curve can be seen in the same figure. Again the Vycor extracts of ACTH-free plasma per se did not influence the bioassay standard curve at all, in contrast to the extract obtained with silicic acid. The results described in fig IV.2 show that Vycor glass powder is to be preferred to the silicic acid or Quso preparation for the extraction of ACTH from plasma. Furthermore, the effectiveness of extraction of unlabelled ACTH was analysed. Increasing doses of ACTH were added to ACTH-free 4 ml plasma samples. After ACTH extraction the ACTH contents of these extracts were analysed by radioimmunoassay and bioassay and the responses (% bound or corticosterone production) were plotted against the amount of ACTH theoretically present in 0.1 ml of reconstituted extracts, which had been added to the assay tubes. In this way we obtained real processed standard curves. These are given in fig IV.2 as well (lines through the black squares). As far as the Vycor extraction is concerned the figure shows that both in the radioimmunoassay and in the bioassay the processed standard curves parallel exactly the normal i.e. the non-processed standard curves. This implies that the recovery of ACTH over a range of values is the same.
In another experiment very high doses of ACTH - up to 4000 pg/ml - were observed to yield recoveries equal to those in the low doses.

With respect to the recovery loss of ACTH has been tested by addition of tracer and unlabelled standard to different volumes of ACTH-free plasma. When processing tracer ACTH the recovery was about equal in plasma volumes up to 7 ml. This is illustrated in fig IV.3. Addition of 200 pg synthetic $hACTH^{1-39}$ to different volumes of ACTH-free plasma (1-4 ml) showed similar recoveries for immunological ACTH for each of the volumes used as well as for biological ACTH. It appeared however in these experiments that the

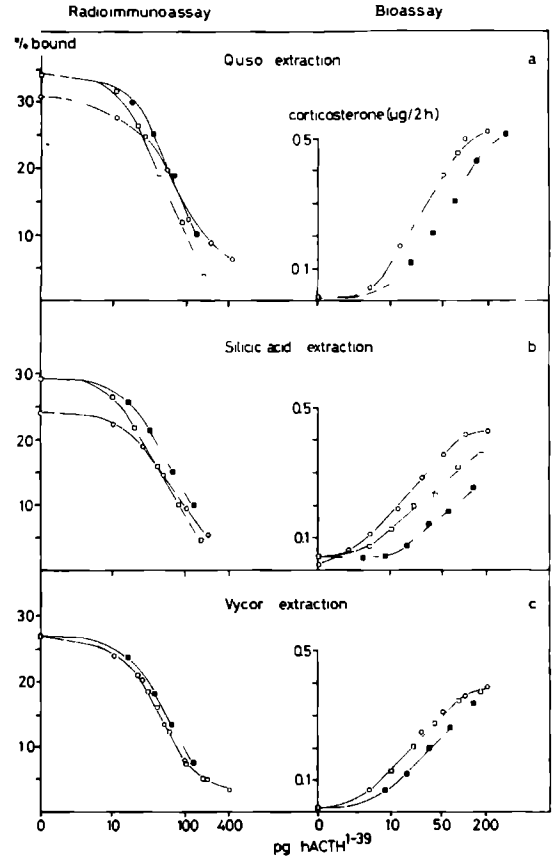

fig IV.2 *The influence of plasma extracts using different glass powders upon the standard curves in radioimmunoassay and bioassay. Vycor and silicic acid extraction was performed as is described in materials and methods. Quso extraction is described in fig* IV.1. *Graded doses of ACTH were added to dried extracts of 4 ml ACTH-free plasma samples, after which 0.1 ml aliquots were transferred to assay tubes (□——□); graded doses of ACTH were also added before the extraction to 4 ml ACTH-free plasma samples; after the extraction 0.1 ml aliquots of the reconstituted extracts were assayed, resulting in a processed standard curve (■——■). Both curves were compared with a non-processed standard curve (○——○).*

recovery of bioactive ACTH was statistically significantly lower ($P<0.05$) than the recovery of immunoreactive ACTH. Measuring the recoveries of immuno-

reactive ACTH and bioactive ACTH concomitantly in ACTH-free plasma pools in 50 experiments over a period of about 4 years, loss of B-ACTH was again found to be significantly greater than the loss of I-ACTH ($P<0.001$; fig IV.4).

In order to get information about the recovery of ACTH in different patient plasma samples, tracer was added to 10 different plasma samples of 4 ml. As is shown in fig IV.5 the percentages of adsorption and recovery did not vary greatly (adsorption loss: 9.4 $\pm$ 1.8%; recovery 87.3 $\pm$ 2.2%). The losses in the wash

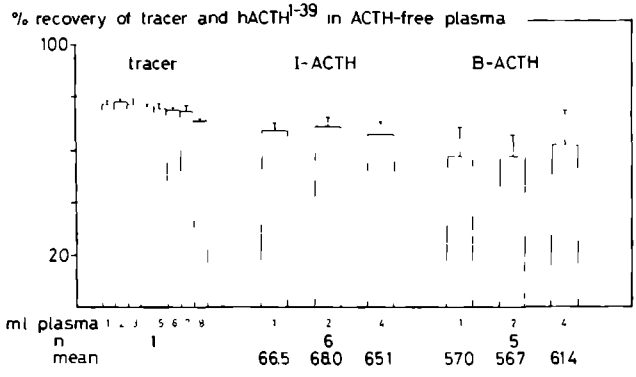

fig IV.3 *The recovery of tracer and standard ACTH in different volumes of ACTH-free plasma as measured by radioactivity, radioimmunoassay and bioassay respectively. 5000 Cpm of the tracer and 200 pg hACTH$^{1-39}$ were added in duplicate in each of the experiments.*

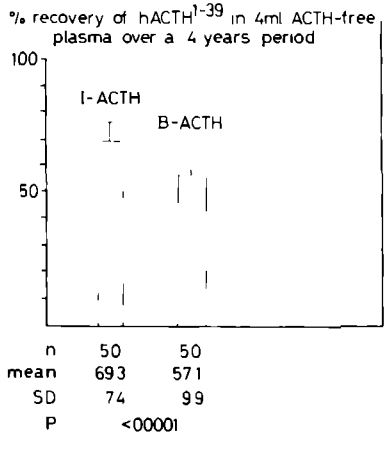

fig IV.4 *The percentage of standard ACTH, added to 4 ml ACTH-free plasma, as measured by radioimmunoassay and bioassay.*

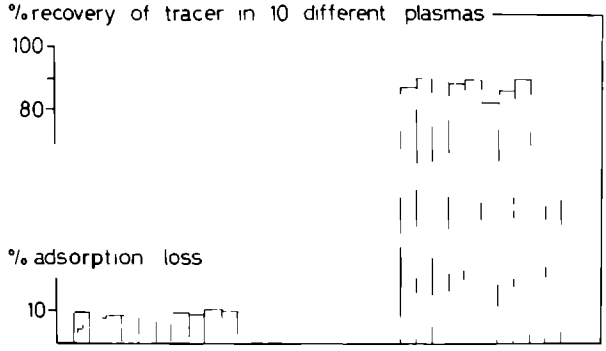

fig IV.5 *The percentage recovery of tracer ACTH in different plasma samples. 10,000 Cpm tracer was added to 4 ml plasma samples from 9 different patients and to 4 ml of ACTH-free plasma (P). The values are the mean of duplicate estimations.*

steps with distilled water and HCl were neglectable. Recovery in ACTH-free plasma and patient plasma was also tested with unlabelled ACTH. In 5 individual experiments with 12 different patient plasma samples the recoveries of ACTH in patient plasma as measured in the radioimmunoassay were found to be virtually the same as the recoveries in ACTH-free plasma (table IV.3). The same was observed when the recoveries in the bioassay were measured. The table also shows that between the experiments individual resurgences vary considerably especially in the bioassay. Therefore the use of a processed standard curve measuring the recovery in each experiment is obligatory.

Plasma extraction by means of glass particles is generally used nowadays to measure plasma ACTH in the radioimmunoassay. Data about recoveries as measured in the radioimmunoassay, however, are often incomplete. Usually recoveries of I-ACTH are reported when standard ACTH had been extracted from ACTH-free plasma. Only - as far as we know - Ichikawa et al (1971), using silicic acid, reported recoveries of I-ACTH in different patient plasma specimens (94 $\pm$ 8%). Recoveries of I-ACTH in ACTH-free pool plasma varying from 42.1 $\pm$ 3.8% to 76% were found by Croughs et al (1973) and Landon & Greenwood (1968), both using Fuller's earth as the extractant. By means of 35 mg Vycor, Ratcliffe & Edwards (1971) found a mean recovery of 58 $\pm$ 10%. Other authors reported the recovery of tracer ACTH in different patient plasma samples: Donald (1967): 78 $\pm$ 0.66% (n=16) and Liotta & Krieger (1975): 72-76% (n=24).

Table IV.3

Recovery of $hACTH^{1-39}$ in 12 different patient plasma samples as compared with the recovery in ACTH-free plasma in the same experiments

experiment	plasma sample[†]	% recovery I-ACTH	% recovery B-ACTH
1	ACTH-free	78	60
	patient a	70.5	54
	" b	72	59
	" c	84	52
	" d	75	49
2	ACTH-free	68	50
	patient e	68	
	" f		41
3	ACTH-free	80	37
	patient g	80.5	47
	" h	76	37
	" i	78.5	47
4	ACTH-free	85	36
	patient j	69	37
	" k	88	38
5	ACTH-free	88	43
	patient l	96	55
	" m	84	42.5

[†] To each plasma samples 50 pg $hACTH^{1-39}$ /ml plasma was added. 11 Patient plasma samples contained 30-122 5 pg I-ACTH/ml and 8-53 pg B-ACTH/ml One sample contained 922 pg I-ACTH/ml and 363 pg B-ACTH/ml plasma.

Data about measurements of bioactive ACTH in human plasma are relatively scarce in literature. Ney et al (1963), using resin column chromatography for ACTH extraction and the Lipscomb-Nelson bioassay - reported a mean recovery of 102 $\pm$ 41% in 11 runs in ACTH-free plasma. Cowan (1976), using glass particles of a commercially available radioimmunoassay kit, reported a recovery of 4.6 $\pm$ 0.3% in an isolated adrenal cell bioassay in human plasma. Liotta & Krieger (1975), however, using silicic acid as the extractant and isolated adrenal cells, found a considerably higher recovery; addition of different doses of standard to ACTH-free plasma resulted in recoveries ranging from 64 to 76% in one experiment. These recoveries were virtually the same as those of tracer ACTH in the same experiment. Due to the lack of data on the recovery of immunoreactive ACTH a possible deactivation of biological activity during the extraction cannot be concluded from their study. Neither are there any data available about the recovery of B-ACTH in different plasma specimens. In our study the mean recovery of B-ACTH as measured over a period of 4 years appeared to be reliable, though lower than the recovery of I-ACTH, indicating a small loss of bioactivity during the extraction procedure (fig IV.4). Furthermore this study shows that the

recoveries are virtually the same in ACTH-free plasma as in patient plasma both for B-ACTH and for I-ACTH (table IV.3).

In order to obtain information about the behaviour of the standard during the extraction procedure in comparison to that of native ACTH, recoveries of synthetic $hACTH^{1-39}$ and of a human pituitary extract were evaluated in two experiments with 10 plasma samples obtained from different patients. Neither in the radioimmunoassay (recovery standard $hACTH^{1-39}$: 86.4 $\pm$ 8.6%; human pituitary extract: 88.6 $\pm$ 7.8%) nor in the bioassay (recovery $hACTH^{1-39}$: 44.8 $\pm$ 8.0%; human pituitary extract. 43.6 $\pm$ 11.5%) discrepant results were obtained.

Besides recovery loss plasma extracts may interfere in the radioimmunoassay as regards to the non-specific binding in a different way as compared to the non-specific binding of the extracts of ACTH-free plasma. Therefore the variability in the non-specific binding of 30 different plasma extracts was investigated, according to the method as described in chapter III. In these experiments the non-specific binding of the plasma extracts obtained from different patients as well as the non-specific binding of the extracts of ACTH-free plasma had been measured. The ACTH values in the plasma samples were calculated twice: both with a correction for the non-specific binding of the ACTH-free plasma extract and with a correction for the non-specific binding of the patient plasma extracts itselves. The two series of ACTH values obtained were compared with each other. The differences appeared to be low as is shown by a low coefficient of variation: 1.38%.

IV.3.2 Further validation of the assays for measurement of human plasma ACTH

IV.3.2.1 Reproducibility

Reproducibility was assessed by calculating the intra-assay and inter-assay duplicate variations both for the bioassay and for the radioimmunoassay for different concentration ranges of ACTH. The mean intra-assay variation for I-ACTH ranged from 6.3% to 6.5% and for B-ACTH from 7.2% to 10.9%. The mean inter-assay variation ranged from 10.4% to 12.3% for I-ACTH and from 10.5% to 14.9% for B-ACTH (table IV.4). Fig IV.6 illustrates the replicate inter-assay variation that occurred when a pooled plasma was measured 10 times in a period of 6 months. The coefficient of variation was calculated to be 11.1% for I-ACTH and 20.5% for B-ACTH.

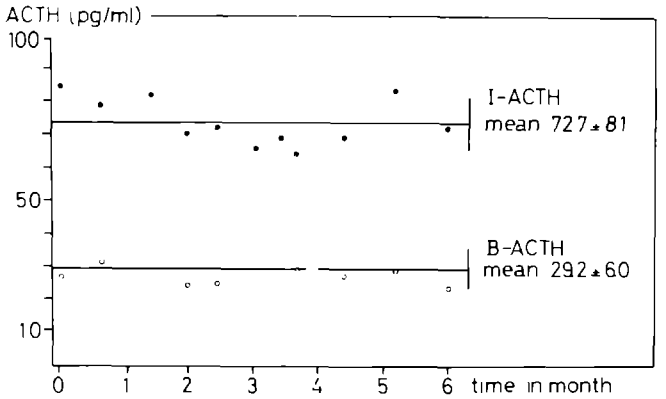

fig IV.6 *The replicate inter-assay variation in a patients plasma pool.*

Table IV.4

Reproducibility of the assays

	Radioimmunoassay			Bioassay		
	range of ACTH levels in the plasma samples/ml			range of ACTH levels in the plasma samples/ml		
	<100 pg	<1000 pg >100 pg	>1000 pg	<100 pg	<1000 pg >100 pg	>1000 pg
Intra-assay duplicate variation						
n	40	40	40	40	40	40
coefficient of var.	6.3	6.4	6.5	10.9	7.2	10.5
Inter-assay duplicate variation						
n	50	30	25	40	20	10
coefficient of var.	12.3	11.1	10.4	14.9	10.5	14.0

IV.3.2.2 Specificity

Amongst other conditions (chapter II, III) a prerequisite for specificity is that the estimated ACTH values in the plasma extracts decrease linearly with dilution of the extract (Berson & Yalow 1968); i.e. there should be a lack of interference by substances other than ACTH or ACTH-like peptides.

Because of a limited sensitivity the specificity could only be extensively evaluated when plasma samples with high ACTH levels were used. In the bioassay as well as in the radioimmunoassay (fig IV.7) a parallelism is shown between on the one hand a dilution of an extract of ACTH-free plasma to which 2000 pg standard had been added and a diluted extract of plasma of an Addison

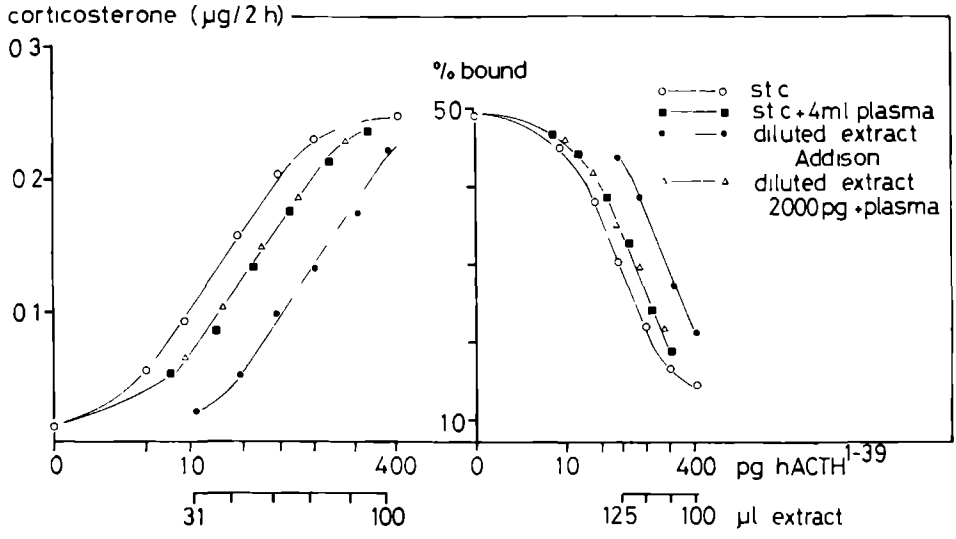

fig IV.7 *Parallelism of two types of processed standard curves - graded doses added to 4 ml ACTH-free plasma samples (■——■), and diluted extracts of 4 ml ACTH-free plasma samples to which 2000 pg standard had been added (△——△) - and a diluted extract of a plasma specimen of a patient with Addison's disease (●——●) with the non-processed standard curve (o——o) both for bioassay and for radioimmunoassay.*

patient, and on the other hand the non-processed standard and the true processed standard curve.

The specificity of the assays for lower ACTH values was approximated by using 1:1 and if possible 1:3 dilutions. Table IV.5 shows that the ACTH values calculated from such diluted extracts from different patient plasma specimens were virtually the same.

IV.3.2.3 Sensitivity

Unknown samples can only be estimated from the linear part of the standard curve. In the bioassay and in the radioimmunoassay the lowest values to be observed reliably from the standard curves amount to about 3 and 10 pg respectively. Assuming a mean recovery of 57% (fig IV.4) and measuring 40% of a 90% eluate of 4 ml plasma, a quantity of about 3 x 100/40 x 100/90 x 100/57 x 1/4 = 3.5 pg/ml plasma could routinely be detected by the bioassay. In the radioimmunoassay - with a mean recovery of 69% - this value is 10 pg/ml.

Table IV.5

Parallelism in radioimmunoassay and bioassay with diluted extracts[†] of different volumes of plasma of different patients

	plasma	I-ACTH in pg/ml plasma				plasma	B-ACTH in pg/ml plasma		
pat.	in ml	0.1ml	0.05ml	0.025ml	pat.	in ml	0.1ml	0.05ml	0.025ml
1	4	25	25		29	4	4	5	
2	4	50	45	48	30	4	24	27	32
3	4	37	34	38	31	4	8.5	11	9
4	4	39	37		32	4	3.5	4	
5	4	74	64	84	33	4	3.5	3.5	
6	4	300	325	313	34	4	7	9	
7	4	61	58		35	4	86	70	
8	4	131	113	105	36	4	17.5	16	
9	4	144	148	143	37	4	11	10.5	
10	4	43	43	40	38	4	8	8.5	
11	3.5	66	61	69	39	4	4.5	6	
12	3.5	47	46		40	4	6	6	
13	3.5	161	123		41	3	6	8	
14	3.5	27	34		42	3	67	63	73
15	3	56	50		43	3	12.5	15	16
16	2.5	174	184		44	3	7	9	
17	2	110	120		45	3	8	9	
18	2	513	650		46	3	25	29	23
19	2	124	138		47	2	45	39	44
20	2	63	70		48	2	33	40	38
21	2	71	74		49	2	29	28	32
22	2	61	68		50	2	65	65	60
23	2	500	436		51	2	23	24	24
24	2	137	144		52	2	40	44	
25	2	69	73		53	2	16	17	
26	2	174	153						
27	1.5	155	157						
28	1.5	146	158						

[†]Extracts were dissolved in 0.25 ml ACTH-diluent

IV.3.3 Choice of standard

A lot of different ACTH standards have reportedly been used in literature (table IV.6 and IV.7).

In this study a number of ACTH standard preparations available from different sources have been compared. In both assays each of these preparations were analysed by their response curves and the quantities of the different preparations - on weight basis - were related to the response of 100 pg $hACTH^{1-39}$. The I-ACTH to B-ACTH ratio (I/B ratio) of $hACTH^{1-39}$ was therefore fixed at 1.00. The data of 5 standard preparations are summarized in table IV.8. All standards showed a parallelism with each other both in the radioimmunoassay and in the bioassay. In the radioimmunoassay the activity of the IWS is 1.8 times as low as compared to the activity of the synthetically prepared standard $hACTH^{1-39}$. It is of importance to note that when the IWS

Table IV.6

Comparison of 18 bioassays for the determination of plasma ACTH

Authors	bioassay	extraction procedure	standard	sensitivity /ml plasma	control morning plasma levels /ml plasma		
					n	mean	range
Sydnor et al (1953)	ascorbic acid depletion	oxycellulose					<5 µU
Fujita (1957)	idem	idem			27	10 µU	22 not detectable
Lipscomb & Nelson (1962)	Lipscomb-Nelson assay	-					not detectable
Vance et al (1962)	idem	-		4 µU	13	7 µU	4- 10 µU
Cooper & Nelson (1962)	idem	-			10		- 9 µU
							7 not detectable
Ney et al (1963)	idem	carbox resin	2nd Int St.		13	2 5µU	1- 6 µU
Espiner et al (1963)	modified L & N assay in sheep	-					2- 3 µU
Davies (1963)	ascorbic acid depletion	oxycellulose	2nd Int St		8	7 µU	5- 12 µU
Vernikos-Danellis et al (1966)	adr corticost. content	-		5 µU	6	9 5µU	6 5-12 5µU
Lefkowitz et al (1970)	radioreceptor	-	Lerner-Upton†	5 pg	7		- 88 pg
Binoux et al (1971)	Lipscomb-Nelson assay	-		1 1 µU	9		1.1-1 4 µU
							7 not detectable
Wolfsen et al (1972)	radioreceptor	-	pur pACTH	10 pg	12	75 pg	12-100 pg
Holdaway et al (1973)	cytochemical assay	-	3rd Int.St	<<1 pg	10	28 pg	12- 42 pg
Daly et al (1974)	idem	-	3rd Int St	<<1 pg		70 pg	
Fleisher et al (1974)	idem	-	3rd Int St	<<1 pg	18	60 pg	24-110 pg
Raux et al (1975)	Lipscomb-Nelson assay	-	3rd Int St			1 6µU	
Liotta & Krieger (1975)	isolated adr cells	silicic acid	3rd Int St.	2 pg	6	32 pg	15- 50 pg
present study	idem	Vycor	synth hACTH	3 5 pg	17	15 pg	5 5- 36 pg

† highly purified human ACTH

Table IV.7

Comparison of 21 radioimmunoassay techniques for the determination of plasma ACTH

Authors	extraction procedure	coeff. of var. intra-assay	coeff. of var. inter-assay	standard	sensitivity pg/ml	control morning plasma levels in pg/ml n	mean	range
Felber (1963)				pur.pACTH	1000			3,000-25,000
Yalow et al (1964)	acetone/HCl			pur.hACTH	25	9	260	150-380
Demura et al (1966)	acetone/HCl	11		pur.pACTH		10	50	30- 70
Berson & Yalow (1968)	-			Lerner-Upton†	1	69	22	-±80
Landon & Greenwood (1968)	Fuller's earth			synth.hACTH††	10	36		12- 55
Besser et al (1971)	"	14	16	Lerner-Upton†		50		15- 70
Matsukara et al (1971)	-	12		synth.hACTH††	10	30	70	22-175
Ichikawa et al (1971)	sil.acid	20		synth.hACTH††		18	46	20- 93
Donald et al (1972)	sil.acid	3.5	16.1	Lerner-Upton†	10	11	41.6	
Galskov (1972)	-	7.6	10.3	synth.pACTH		106	125	60-250
Kendall et al (1973)	Vycor			Lerner-Upton†				15-100
Croughs et al (1973)	Fuller's earth		14	Lerner-Upton†	7	23		-±60
Genazzani et al (1974)	Vycor	11.3	16	synth.hACTH††	10	14	17	7.5-28
Voigt et al (1974)	Quso			pur.pACTH		44	30	-120
Horgan & Riley (1974)	Fuller's earth	5.1	13.6	synth.hACTH††	6	50		6- 68
Fleisher et al (1974)	Vycor			Lerner-Upton†	30	18	72	30-140
Liotta & Krieger (1975)	sil.acid	5.6	16.9	3rd Intern.St.		4	34	19- 50
Kao et al (1979)	-	7.6	10.6	pur.hACTH		103	41	-136
Krieger et al (1979)	sil.acid					24	88	30-156
Thoren et al (1981)	-	12.1	21	synth.hACTH×	20	39	147	81-270
present study	Vycor	6.4	11.3	synth.hACTH×	10	17	49	16-127

† highly purified human ACTH preparation

†† obtained from Gedeon Richter

× obtained from CIBA-Geigy

Table IV.8

Quantities of standard preparations as compared to the activity of 100 pg synthetic $hACTH^{1-39}$ in radioimmunoassay and bioassay

Standard	pg standard in radioimmunoassay	pg standard in bioassay	I/B ratio relative to the ratio of $hACTH^{1-39}$
$hACTH^{1-39}$	100	100	1 00
hum.hyp.extract	98	212.5	0.46
3rd Intern.St.(IWS)†	180	365	0.49
Cortrophin†	240	275	0 87
$ACTH^{1-24}$	65	12.5	5.20

† pituitary extract of porcine origin

would be used as the standard the values of unknown samples would be 1.8 times as high as the values recorded in this thesis which were obtained using the synthetic $hACTH^{1-39}$ as the standard. Besides the IWS another porcine ACTH preparation, Cortrophin, showed lower immunoreactivity than the standard $hACTH^{1-39}$. This suggests the presence of other than ACTH-like material in these pituitary extracts. This is the more striking, when the biological activity of these extracts are compared to $hACTH^{1-39}$.

Using the IWS as standard in the bioassay the values of unknown samples will be scored 3.65 times higher than the values obtained using the synthetic $hACTH^{1-39}$ as the standard. The activity of a human pituitary extract was almost identical to that of the standard in the radioimmunoassay but showed a lower activity in the bioassay than the standard $hACTH^{1-39}$. This might indicate that some ACTH-like material present in this human pituitary extract evinces crossreactivity with the ACTH antibody but is devoid of high steroidogenic potency.

Of course the choice of the standard has large implications not only on I-ACTH and B-ACTH values but also on the I/B ratio. In the most extreme cases first - when $ACTH^{1-24}$ would be used in the radioimmunoassay and the IWS in the bioassay - an I/B ratio about 0.18 would be obtained, secondly - when Cortrophin would be used in the radioimmunoassay and $ACTH^{1-24}$ in the bioassay - an I/B ratio of about 20 would be observed. This has to be considered when I/B values of different laboratories are compared.

In this study it was chosen to calibrate I-ACTH and B-ACTH values against the same standard. The choice of standard was synthetic $hACTH^{1-39}$ for a number of reasons:

- it contains the human ACTH amino acid sequence

- it is more stable than ACTH$^{1-24}$
- it is not a pituitary extract, which might contain ACTH precursor or ACTH-like side-products
- it is widely available (it is distributed now by the National Pituitary Agency of the U.S.A.).

IV.3.4 Normal plasma ACTH levels at 900 h: radioimmunoassay versus bioassay.

The plasma ACTH levels of 17 healthy subjects measured by both assays are depicted in table IV.9. In each subject immunoreactive ACTH was higher than the bioactive value, which results in an I-ACTH to B-ACTH ratio (I/B ratio) higher than unity. It should be noted that in both males and females the range of the I/B ratio was rather wide (2.00 to 6.30). However, when the I-ACTH values are compared with the B-ACTH values a correlation is observed ($r=0.874$) which reaches a statistical significance ($P<0.001$). In spite of the observed dichotomy between the I-ACTH and B-ACTH levels this high correlation indicates the usefulness of the radioimmunoassay in normal subjects.

Table IV.9

Plasma ACTH levels in 17 normal subjects at 900 h

	Immunoreactive ACTH pg/ml	Bioactive ACTH pg/ml	I-ACTH/B-ACTH ratio
males			
1	63	10	6.30
2	83	30	2.77
3	48	15.5	3.10
4	57	16.5	3.45
5	19	9.5	2.00
6	80	19.5	4.10
7	57	11.5	4.96
8	30	10.5	2.86
9	127	36	3.53
10	38	14	2.71
11	24	11	2.18
12	69	29	2.38
females			
13	27	9.5	2.84
14	18	5.5	3.27
15	33	8	4.13
16	16	6	2.67
17	39	15.5	2.52
mean	48.7	15.1	3.28
SD	29.4	8.8	1.09

As far as the question is concerned why these immunoassayable ACTH values exceed the bioassayable values several causes might contribute to this phenomenon. The specificity study in chapter II revealed that in the bioassay only ACTH moieties which contain both the bioactive center and the receptor binding sequence evoked a biological response in a physiological range. In the radioimmunoassay (chapter III) an antibody is used which recognizes the midportion sequence (17-24) of the ACTH molecule which is virtually devoid of a biological response. This certainly could result in an overestimation of ACTH by radioimmunoassay, because it is possible that different ACTH-like peptides occur with an intact sequence (17-24). This suggestion is in accordance with the observations of Bennett et al (1977) who reported that peptide inactivation occurs as a result of catabolism by peptidases. Their high pressure liquid chromatography disclosed such peptide fragments. There is, however, according to these investigators another important route for the inactivation of ACTH. From their results they conclude that sulphoxidation converts ACTH to a product that has very low biological activity. As this sulphoxidation concerns only methionine, the amino acid localized in position 4 of the ACTH molecule, it is highly probable that the product has immunoreactivity. This suggestion is in accordance with the results obtained by Nicholson et al (1978) who observed transformation of injected radioactive methylated $hACTH^{1-39}$ to a product which remained immunoreactive and is described to loss biological activity by a rather subtle alteration at the N-terminal sequence.

Other possible explanations for the observed higher I-ACTH as compared to B-ACTH values are less likely:

i) interferences of plasma substances in the assays. As was shown above no such interferences were observed in the radioimmunoassay or in the bioassay (fig IV.7; table IV.5).

ii) the standard employed. As was stated in section IV.3.3 the use of a particular standard could be influence the I/B ratio. However, when a standard with a lower I/B ratio is used - the human hypophyseal extract (table IV.8) - the I-ACTH values calculated are still higher than the B-ACTH values.

iii) interferences of ACTH-related peptides in the assays. In this respect a possible presence of the socalled "big ACTH" (section I.2) in the circulation could be detected in the radioimmunoassay but not in the bioassay (Gewirtz et al 1974). However "big ACTH" is thus far detected in plasma without the Vycor extraction procedure. Nothing is known about the

extractibility of "big ACTH" by using Vycor glass powder. Moreover, using a chromatographic procedure which prevents the non-specific binding of ACTH to plasma proteins, Ratter et al (1980) found no "big ACTH" present in plasma of normal subjects, patients with pituitary-dependent Cushing's syndrome, Addison's disease and Nelson's syndrome but did find "big ACTH" in the plasma of patients with an ectopic ACTH syndrome. For these reasons it is not likely that "big ACTH" was measured in one of our assays. As ACTH-related peptides did not influence the bioassay (β-endorphin: section II.3.3.2.1) or even enhanced ACTH-induced steroidogenesis (16K fragment, γ-MSH: Pedersen & Brownie 1980; Pedersen et al 1980; Al-Dujaili et al 1981), the activity of these peptides would not influence the I/B ratio or even cause a low I/B ratio. Only two reports exist comparing I-ACTH with B-ACTH. Fleisher et al (1974) who used the cytochemical assay as the bioassay found a mean I/B ratio (1.20) which was much lower than in our study, but they used different standards in each assay. As a bioassay system Liotta & Krieger (1975) used trypsin dispersed adrenal cells derived from hypophysectomized rats and they found I/B ratios varying from 1.10 to 1.39 using the 3rd International Standard as the standard. It has to be emphasized that using this standard in our assays the I/B ratio amounted to about 1.60, a value similar as found by Liotta & Krieger (1975). Furthermore it is of interest that for other hormones immunoreactivity and bioactivity may also show a dichotomy (Solano et al 1979; Robertson et al 1979; Marana et al 1979; and Mukhopadyay et al 1979 for LH, Segre et al 1972 for PTH, Leung et al 1978; and Asawaroengchai & Nicoll 1977 for prolactin, Roos et al 1978 for calcitonin).

The mean morning plasma I-ACTH value and its range are in close agreement with most of the recently reported values (table IV.7). Where differences still exist in the reported values these may be due to the use of different antibodies detecting different ACTH metabolites (chapter VI), or to the use of different standards (vide supra) or to different extraction procedures.

Before the development of a radioimmunoassay for ACTH, bioassays were performed with a relatively low sensitivity which required a large amount of plasma and time consuming extraction procedures (Ney et al 1963; Davies 1963). Using the ascorbic acid depletion test and 200 ml plasma Fujita (1957) detected ACTH activity in only 5 out of 27 subjects, whereas Binoux et al (1971), who used the Lipscomb-Nelson assay, did find detectable ACTH activity in 2 out of 9 subjects. Table IV.6 summarizes the normal values obtained by several authors using different techniques. Seven of the authors listed performed bioassays using steroidogenic activity of ACTH. Liotta & Krieger (1975) were the first

who presented data about the ACTH bioactivity in plasma of normal human subjects as measured in an isolated rat adrenal cell assay after glass powder extraction. The reproducibility of their assay was similar to that of our study. They reported an intra-assay and an inter-assay coefficient of variation of 9.8% and 12.7% respectively (cfr table IV.4). The B-ACTH values reported in our study are somewhat lower than those in other reports (table IV.6).

Table IV.10

I/B ratio's in 8 normal subjects at 4 times on the same day

Subject	900 h	930 h	1600 h	1630 h
1	2.00	2.38	2.12	2.31
2	4.96	3.47	2.28	3.33
3	3.27	3.40	2.74	2.16
4	3.53	3.77	2.57	2.04
5	2.71	2.61	2.35	2.76
6	4.13	2.88	4.66	5.00
7	3.02	3.34	2.15	4.03
8	3.11	2.32	2.12	2.50
mean	3.34	3.02	2.62	3.01
SD	0.90	0.55	0.85	1.04

This may be due to the use of a different standard. For instance, a B-ACTH value almost similar to that reported by Fleisher et al (1974) could be calculated from our data when the International Working Standard was used as a standard (cfr tables IV.6 and IV.8).

As the I/B ratio varied widely interindividually - from 2.00 to 6.30 in our study - we studied the intra-individual change in I/B ratio at four sets of time during the day in 8 normal subjects (table IV.10). No statistically significant variations between the mean I/B ratios could be observed at the different sampling times.

BIOACTIVE VERSUS IMMUNOREACTIVE ACTH IN PLASMA: CLINICAL STUDIES

V.1 Introduction

Before a sensitive radioimmunoassay was available (Berson & Yalow 1968) the assessment of the pituitary-adrenal gland function was hampered by the inherent insensitivity of bioassays requiring large samples of blood (chapter IV). Therefore the number of reports dealing with bioassayable ACTH levels in normal subjects and patients with disturbances in the pituitary-adrenal axis is relatively small (cfr Ney et al 1963; Liddle et al 1962) compared to the numerous studies in which ACTH was measured by radioimmunoassay. Only very few reports exist which compare immunoreactive and bioactive ACTH levels (Fleisher et al 1974; Liotta & Krieger 1975). In both these studies higher ACTH levels were detected by immunoassay than by bioassay. The availability of a very sensitive bioassay as described in this thesis enabled us to compare immunoreactive ACTH (I-ACTH) and bioactive ACTH (B-ACTH) in plasma in normal and pathological conditions, as in pituitary-dependent Cushing's disease, Nelson's syndrome and Addison's disease.

V.2 Materials and Methods

Subjects studied

Control subjects. In order to delineate normal ranges for I-ACTH, B-ACTH and cortisol, plasma was obtained from 17 laboratory collaborators - 12 men and 5 women (mean age: 31.1 $\pm$ 6.7 year, range 19 to 43 year) - and from 23 hospitalized patients without clinical and chemical evidence of pituitary-adrenal axis dysfunction. This group included 3 men and 20 women (mean age: 27.8 $\pm$ 10.7 year, range 16 to 48 year).

Patients with Cushing's disease. In 21 hospitalized patients with pituitary-dependent Cushing's disease the profile of the pituitary-adrenal axis was studied: 18 female patients (mean age: 41.6 $\pm$ 12.2 year, range 18 to 65 year) and 3 male patients (age 24,56 and 59 year).

Five patients were studied after bilateral adrenalectomy without substitution for 24 hours: 4 female patients (mean age: 37.5 $\pm$ 19.1 year, range 18 to 62

year) and 1 male patient (age 55), and 9 other patients on substitution (25 - 37.5 mg hydrocortisone acetate and 0.1 to 0.2 mg α-fluorohydrocortisone at 800 h): 6 women (mean age: 44.8 ± 8.0 year, range 30 to 51 year) and 3 men (age 24, 37 and 59 year). Nine patients were studied before as well as after total adrenalectomy.

Patients with Nelson's syndrome. Three female patients with proven Nelson's syndrome (age: 35, 36 and 41 year) also participated in this study. These patients had been bilaterally adrenalectomized for Cushing's disease between 9 and 18 years previously.

Patients with Addison's disease. Ten hospitalized patients with proven adrenocortical insufficiency were studied: nine male patients (mean age: 45.1 ± 16.6 year, range 24 to 61 year) and one female patient (61 year). With the exception of one male patient who had had a deoxycorticosterone acetate (DOCA) implantation, all patients had received their last substitution - hydrocortisone as the glucocorticoid and 9 α-fluorohydrocortisone as the mineralcorticoid - the day before blood was sampled. One patient had a "Sertoli cell only" syndrome in addition. This patient will be discussed separately.

Clinical studies

Basal values at 900 h. From all subjects studied blood was obtained between 800 and 1000 h. From some patients with Cushing's disease, Addison's disease and from the three patients with Nelson's syndrome blood was collected on several days and therefore their basal value was calculated as the mean of these different values. Individual values were employed only in calculations of the correlation coefficients between I-ACTH and B-ACTH or between ACTH and cortisol.

Circadian variation. 1. Blood was obtained at 800 or 900 h, 1200 h, 1600 h, 2000 h, 2400 h and at 400 h respectively from 11 patients without clinical and biochemical evidence of pituitary-adrenal axis dysfunction who volunteered as control subjects, from 13 hospitalized patients with untreated Cushing's disease and from 3 hospitalized patients with Addison's disease without substitution therapy.

2. From 13 laboratory collaborators blood was obtained at 900 h and 1600 h. Plasma I-ACTH and B-ACTH values of these control subjects were compared to those of 15 patients with a normal pituitary-adrenal function, 15 patients with untreated Cushing's disease, and 6 patients with Addison's disease.

Short-term variation. In order to assess the effect of short-term variations blood was sampled twice at 900 h and at 930 h from 13 laboratory collaborators, 11 patients without pituitary-adrenal disorders (three of them on two occasions), and 15 patients with Cushing's disease (four of them on several occasions).
Dexamethasone suppression test. In 10 laboratory collaborators, 11 patients with untreated Cushing's disease and in one patient after bilateral adrenalectomy the effect of dexamethasone was studied: on day 1 blood was collected at 900 h and 1 mg dexamethasone was administered at 2300 h. On day 2 blood was collected at 900 h. On day 3 two mg dexamethasone were administered at 2300 h, after which blood was collected on day 4 at 900 h.

Assays

Bioactive ACTH (B-ACTH) and Immunoreactive ACTH (I-ACTH) levels were determined in plasma samples as described previously. Plasma cortisol levels were determined by radioimmunoassay as described by Smals et al (1978). In short this procedure was as follows. Corticosteroid binding globulin was denaturated by incubation at 70°C for 1 hour after dilution of the plasma with ethanol/water (1:20). The antibody, raised in sheep against cortisol-21-hemi-succinate coupled to bovine serum albumin, and tracer ^{3}H-cortisol were added. After incubation at 4°C for 18 h bound and free hormones were separated by dextran-coated charcoal. Replicate analysis of a plasma pool with a cortisol level of 0.20 µmol/l disclosed intra- and inter-assay coefficients of variation of 5 and 10% respectively. The inter-assay duplicate variations of 8.6%, 7.4% and 4.3% were calculated from determination of samples containing 0.10 - 0.20, 0.20 - 0.30, and 0.30 - 0.60 µmol/l respectively (n=17).

Statistics

Statistical significance between the means of two groups was determined by the Wilcoxon's two-sample test (P). Patterns in rhythmicity were established by way of Friedman's non-parametric analysis of variance (P^{x}), whereas differences in ACTH and cortisol between the different time intervals were statistically assessed by using Wilcoxon's signed rank test (P^{xx}). Correlation coefficients (r) were calculated using Spearman's rank correlation test. Using the χ^2-test the significance was denoted by P^{+}. It has to be noted that in the figures each significance was denoted by P.

V.3.1 Basal plasma levels of Immunoreactive ACTH, Bioactive ACTH and cortisol at 900 h

V.3.1.1 I-ACTH levels

The I-ACTH levels are depicted in fig V.1. The patients without disorders

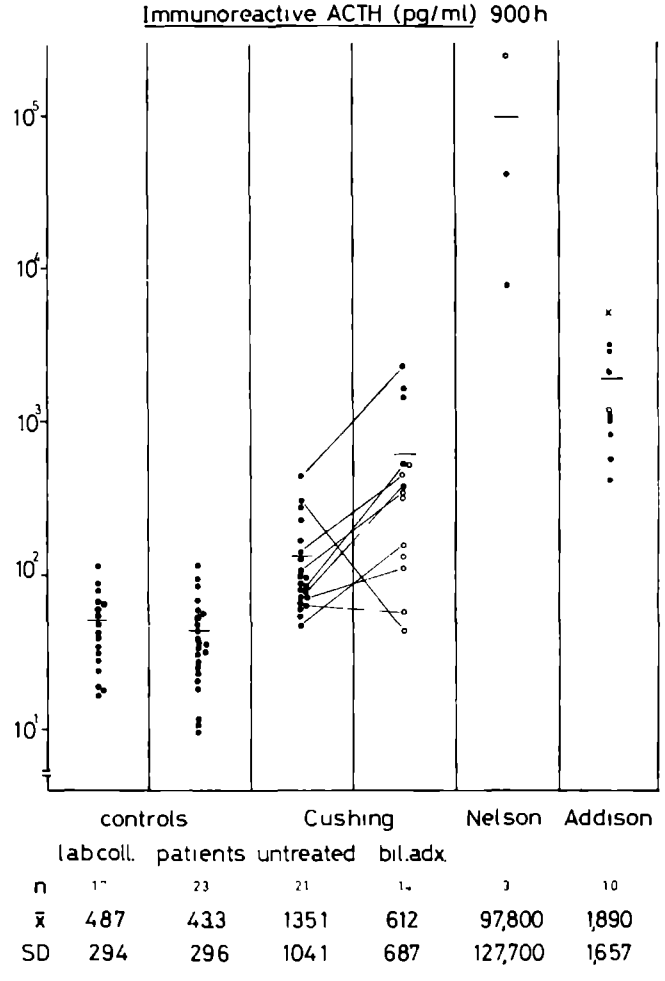

fig V.1 *The immunoreactive ACTH values at 900 h. Open circles indicate patients on substitution therapy. The cross points to the patient with a "Sertoli cell only" syndrome in addition to Addison's disease. Lines connect values of the same patient before and after adrenalectomy.*

of the pituitary-adrenal axis had virtually the same mean I-ACTH value as the laboratory collaborators: 43.3 ± 29.6 pg/ml versus 48.7 ± 29.4 pg/ml. Moreover, the ranges were in close agreement with each other: 10-130 pg/ml versus 16-127 pg/ml. Therefore the "normal" I-ACTH levels at 900 h were somewhat arbitrarily chosen to range from 10-130 pg/ml.

Although numerous data are reported about normal I-ACTH values, only a few reports exist in which I-ACTH values of non-hospitalized normal subjects are compared to those of hospitalized patients with a normal pituitary-adrenal function. Berson & Yalow (1968) pointed out that in hospitalized patients with minor disorders as well as in more seriously ill patients, much higher concentrations - up to 500 pg/ml - were to be found as compared to those in hospital personnel and in hospitalized subjects without serious illness (highest levels of about 80 pg/ml). Horgan & Riley (1974) comparing seriously ill patients and out-clinic patients found a mean I-ACTH value twice as high as in laboratory personnel. At variance with their data we found almost similar basal I-ACTH levels at 900 h in our hospitalized and non-hospitalized control groups.

The I-ACTH values in 21 patients with untreated Cushing's disease ranged from 49.5 to 462 pg/ml. The mean value of 135 pg/ml was significantly higher ($P < 0.001$) than the mean value (45.6 ± 29.3 pg/ml) taking both "normal" populations together. Fourteen of these 21 Cushing patients (67%) showed values overlapping the normal range. This finding confirmed data of Besser & Landon (1968). They studied 20 patients with untreated Cushing's disease and found that in the majority of the patients the I-ACTH levels were within or slightly above their normal range (12-60 pg/ml), whereas only 6 plasma levels were detected above 100 pg/ml. Other reports (Berson & Yalow 1968; Landon & Greenwood 1968; Croughs et al 1973; Voigt et al 1974; Kao et al 1979) also stressed the overlapping of ACTH plasma levels in pituitary-dependent Cushing's syndrome and in normal subjects. Therefore, as is well known, the I-ACTH values at 900 h are of limited value to diagnosis of pituitary-dependent Cushing's syndrome.

In all 5 patients with pituitary-dependent Cushing's disease after bilateral adrenalectomy and off substitution for at least 24 hours I-ACTH levels exceeded those in both control groups (range: 385-2295 pg/ml; mean: 1266 ± 796 pg/ml). This mean value was significantly higher than the I-ACTH level before adrenalectomy ($P < 0.01$). The I-ACTH levels ranged from 45 to 550 pg/ml (mean: 248 ± 186 pg/ml) in the 9 patients who took their substitution therapy about 1 hour before blood sampling in the morning. This value was significantly lower than that in patients off substitution for 24 hours ($P <$

0.02). Three of these patients on substitution therapy had I-ACTH levels in the normal range. When comparing only those 9 patients who were studied before as well as after bilateral adrenalectomy it appeared that in the three patients after adrenalectomy without substitution higher I-ACTH levels are present than before the operation. On regular substitution therapy, 4 patients showed higher and two showed lower I-ACTH levels.

In three patients with classical Nelson's syndrome - bilaterally adrenalectomized 9 to 18 year earlier for Cushing's disease - I-ACTH values ranged from 7,850 to 244,000 pg/ml. In literature a similarly wide range has been reported, from 700 pg/ml (Donnadieu et al 1976) to 100,000 pg/ml (Croughs et al 1977).

In the present study the I-ACTH levels in the plasma of patients with Addison's disease off substitution therapy for 24 hours ranged from 408 to 5,806 pg/ml. The highest value was of the patient with a concomitant "Sertoli cell only" syndrome. The morning levels reported in literature show a similarly large range in patients with Addison's disease varying from as low as 82 pg/ml (Donald et al 1972) to about 8,000 pg/ml (Oliver et al 1971).

V.3.1.2 B-ACTH levels

The B-ACTH levels in the plasma of both control groups ranged from 5.5 to 36 pg/ml (mean 15.1 $\pm$ 8.8 pg/ml) for the laboratory collaborators and from 3.5 to 45 pg/ml (mean 12.5 $\pm$ 11.7 pg/ml) for the hospitalized patients without pituitary-adrenal axis disorders (fig V.2). Both mean values did not differ significantly ($P > 0.10$) and the ranges were also in close agreement with each other. Therefore B-ACTH levels for normal subjects were chosen to range, somewhat arbitrarily, from 3.5 to 45 pg/ml.

The plasma B-ACTH levels in untreated Cushing's disease ranged from 10 to 117 pg/ml. Although the mean value of 33.8 $\pm$ 30.8 pg/ml was significantly higher ($P < 0.001$) than the mean value of both control groups together (mean: 13.8 $\pm$ 10.3 pg/ml), fourteen out of 16 B-ACTH values were in the normal range.

Data comparing B-ACTH levels in Cushing's disease with those in normal subjects are rather controversial. Some authors reported invariably elevated levels whereas others found normal or only slightly elevated levels with a wide overlap with the normal range. Jailer et al (1975) and Segal et al (1970) who used the adrenal weight maintenance assay found corticotropic activity in almost all patients but in none of the normal subjects. A similar finding was reported by Davies (1964) who used the ascorbic acid depletion test. On

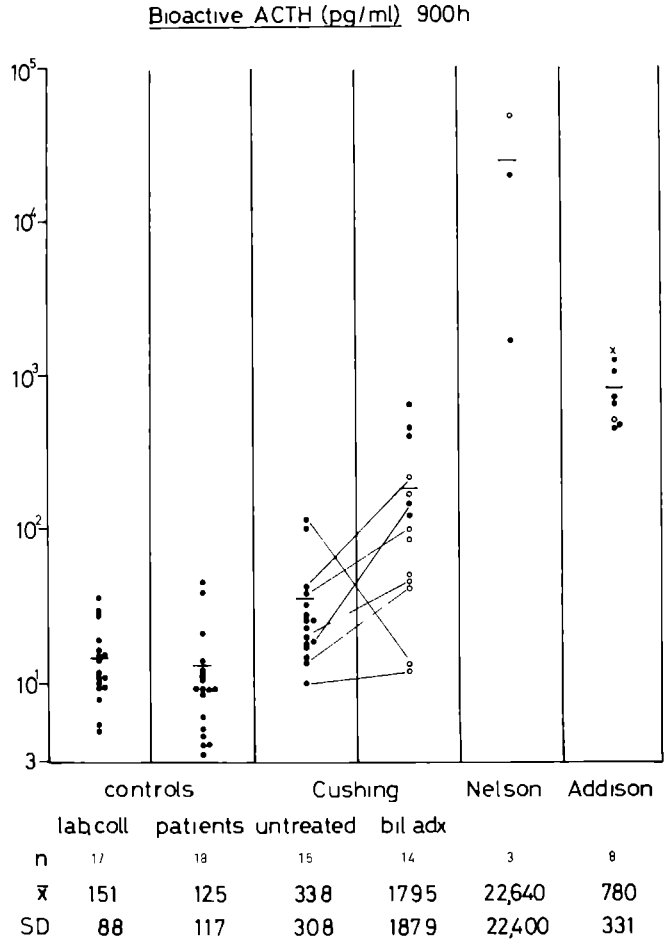

fig V.2 *The bioactive ACTH levels at 900 h. Open circles indicate patients on substitution therapy.* x*: patient with a "Sertoli cell only" syndrome in addition to Addison's disease. Lines connect values of the same patients before and after adrenalectomy.*

the other hand other authors (Vance et al 1962; Liddle et al 1962; Ney et al 1963) who used the Lipscomb-Nelson assay found most of the B-ACTH values of untreated Cushing's disease in the normal range. Using the same assay Raux et al (1975) did not find any difference either between 49 patients with Cushing's disease and 63 controls as regards the B-ACTH levels. Using isolated adrenal cells for measuring B-ACTH and using a multiple sampling procedure in two patients with untreated Cushing's disease Liotta & Krieger (1975) found

B-ACTH levels from 65-210 and 113-200 pg/ml between 900 and 1200 h. In one normal subject sampled between 1000 and 1300 h at 5 min time intervals they reported a range of B-ACTH from 14-93 pg/ml and in 4 additional control subjects from 14.5 to 54.5 pg/ml between 900 and 1000 h. In our studies in which single blood samples were taken at 900 h from 16 untreated patients with Cushing's disease most of the B-ACTH levels were within the normal range.

In the 14 patients treated for Cushing's disease by bilateral adrenalectomy B-ACTH levels ranged from 12 to 637 pg/ml. The mean value of 179.5 pg/ml was significantly higher than the mean of the untreated patients ($P < 0.01$). The B-ACTH levels in the patients on corticosteroid therapy ranged from 12 to 205 pg/ml (mean. 80 $\pm$ 68 pg/ml, $P > 0.10$ versus untreated patients with Cushing's disease),whereas the patients off substitution therapy for at least 24 hours showed B-ACTH levels ranging from 145 to 637 pg/ml (mean: 359 $\pm$ 207 pg/ml), values which were significantly higher than those in untreated Cushing's disease ($P < 0.01$). In 6 out of 7 patients with Cushing's disease B-ACTH levels after bilateral adrenalectomy - 6 patients on and 1 off substitution therapy - were higher than before treatment.

In the three patients with Nelson's syndrome the bioactive ACTH levels were 1670, 19950 and 46288 pg/ml respectively and they were without exception higher than those in treated or untreated patients with Cushing's disease or in patients with Addison's disease. Nelson et al (1958) using the Nelson & Hume (1955) bioassay found similarly high values - up to 4000 µU/ml ($\pm$ 40,000 pg/ml) - as in our study. Using the isolated adrenal cell assay Liotta & Krieger (1975) reported a value of 1029 pg/ml in one patient with Nelson's syndrome, whereas Espinoza et al (1975) using the Lipscomb-Nelson assay reported levels of 280 to 600 µU/ml in 6 patients.

In Addison's disease B-ACTH levels ranged from 450 to 1225 pg/ml. In contrast with I-ACTH, the mean B-ACTH level (780 $\pm$ 331 pg/ml) in the Addison patients was significantly higher ($P < 0.05$) than in the bilaterally adrenalectomized patients with Cushing's disease off substitution therapy (359 $\pm$ 207 pg/ml). Other authors, however, reported somewhat lower B-ACTH values in patients with Addison's disease. Using the classical Lipscomb-Nelson assay Graber et al (1965) found values ranging from 5 to 50 µU/ml and Williams et al (1961) from below 5 to 13 µU/ml. Liotta & Krieger (1975) reported a B-ACTH value of 1028 pg/ml in one patient.

V.3.1.3 The ratio of I-ACTH to B-ACTH

As is shown in fig V.3 the I-ACTH to B-ACTH ratio (I/B ratio) was about the same for both groups of control subjects (3.28 ± 1.09 and 3.47 ± 1.19), although the range was rather wide for both groups (1.39 to 6.30, cfr chapter IV).

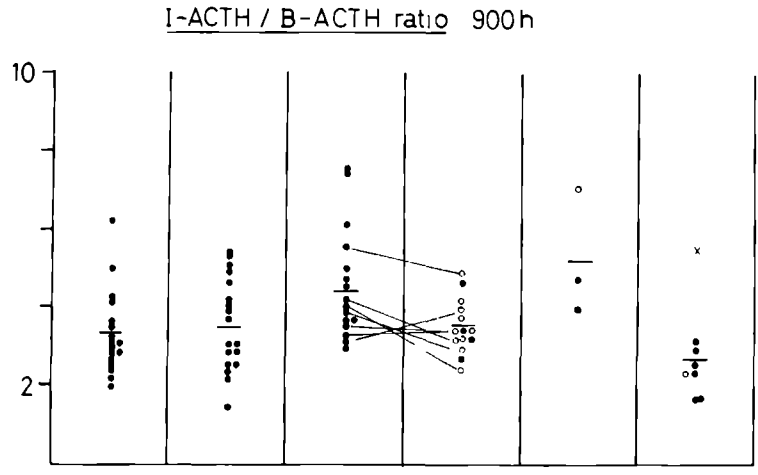

fig V.3 *The I/B ratio at 900 h. Open circles indicate patients on substitution therapy.* x: *patient with a "Sertoli cell only" syndrome in addition. Lines connect values of the same patients before and after adrenalectomy.*

In untreated patients with Cushing's disease the mean I/B ratio (4.40 ± 1.43) was slightly though significantly higher ($P < 0.05$) than in the group of laboratory collaborators, whereas in comparison with patients without pituitary-adrenal axis dysfunction, this difference lacked statistical significance ($0.05 < P < 0.10$). In two patients with untreated Cushing's disease in whom I-ACTH and B-ACTH were measured repeatedly, the I/B ratio - varying from 5.20 to 12.00 - was always higher than 5.00. Only scarce reports have appeared comparing I-ACTH and B-ACTH levels in patients with untreated Cushing's disease. Liotta & Krieger (1975), who used the isolated adrenal

cell system as bioassay, reported mean I/B ratios in two patients with Cushing's disease similar to those of control subjects, whereas Ratter et al (1977), who used the cytochemical assay as bioassay preliminarily reported a mean I/B ratio of 1.1 in 25 patients with pituitary-dependent Cushing's disease against 0.97 in normal subjects. These authors also observed in one patient a much higher I/B ratio ranging from 3 to 13. Taken together, these findings suggest that in a minority of patients with a pituitary-dependent Cushing's disease the physiologically occurring discrepancy between I-ACTH and B-ACTH is even more expressed.

After bilateral adrenalectomy the I/B ratio in the 14 Cushing patients studied was similar to that in both control groups ($P > 0.10$). The I/B ratio in 4 patients off substitution therapy for at least 24 hours (3.49 ± 0.73) was almost identical ($P > 0.10$) to that in patients on corticosteroid therapy (3.51 ± 0.74).

In Nelson's syndrome the I/B ratio ranged from 3.95 to 6.97. Ratter et al (1977) found a mean ratio of 7.8 in Nelson's syndrome, about eightfold higher than their mean control value (0.97). In contrast Liotta & Krieger (1975) reported an I/B ratio of 1.33 in one patient, a value similar to their control value.

The finding of a high I/B ratio in some patients with Cushing's disease and Nelson's syndrome suggests the accumulation of immunoreactive but biologically inactive ACTH fragments or precursors in blood. This relative accumulation of immunoreactive ACTH as compared to bioactive ACTH in the plasma of these patients might be due to the release of ACTH-like peptides by the pituitary, and/or due to differences in the metabolism of ACTH peripherally.

The mean I/B ratio in plasma of patients with Addison's disease (2.71 ± 1.14) was significantly lower than the ratio in patients with Cushing's disease, untreated as well as after bilateral adrenalectomy ($P < 0.001$). Although the ratio in Addison's disease also tended to be lower than in both control groups this difference lacked statistical significance ($0.05 < P < 0.10$). After excluding the patient with the combination of Addison's disease and the "Sertoli cell only" syndrome the mean I/B ratio (2.34 ± 0.56) proved to be significantly lower than the mean value in both control groups ($P < 0.05$). In contrast to our data Liotta & Krieger (1975) reported an I/B ratio of 1.19 in one patient with Addison's disease, a value similar to that found in normal subjects. To illustrate the discrepancies in this field further Ratter et al (1977) preliminary reported an even increased I/B ratio of 4.3 against 0.97

for normal subjects. Our finding of a lowered I/B ratio in the group of 7 patients with Addison's disease, due to the presence of relatively higher amounts of B-ACTH in this disease, may be a physiological response of an essentially normal pituitary - though at a much higher gear - to a failing adrenal gland. The phenomenon of a persistently low I/B ratio in Addison's disease is reminiscent of a similarly lowered I/B ratio observed shortly after insulin-induced hypoglycaemia in control subjects (cfr chapter VI).

V.3.1.4 Cortisol levels

The mean plasma cortisol values in both control groups (0.426 ± 0.102 µmol/l for the laboratory collaborators and 0.362 ± 0.09 µmol/l for the patients without pituitary-adrenal disorders) did not differ significantly, whereas the ranges were similar as well (0.26 - 0.61 and 0.19 - 0.59 µmol/l respectively) as is shown in fig V.4.

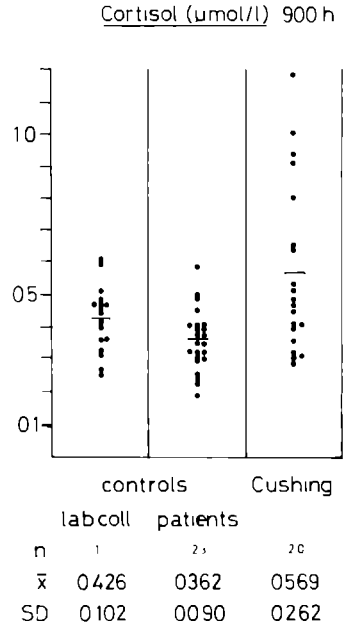

fig V.4 *Plasma cortisol levels at 900 h in control subjects and patients with Cushing's disease.*

The mean plasma cortisol level in the untreated Cushing patients was significantly higher than the mean of the whole control group (0.389 ± 0.100 µmol/l; P <0.01). However, only in 7 out of 20 (35%) patients with Cushing's disease plasma cortisol levels at 900 h a.m. exceeded the normal range.

V.3.1.5 Relations between I-ACTH and B-ACTH levels in plasma

Using the individual basal 900 h values a highly significant correlation was found between I-ACTH and B-ACTH in both groups of control subjects (fig V.5 and V.6; r= 0.874, P <0.001 in the laboratory collaborators, and r= 0.879, P <0.001 in the patient control group).

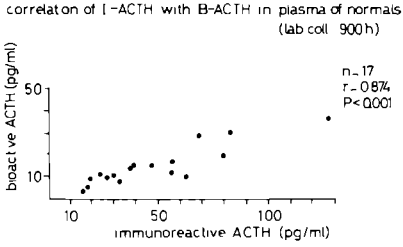

fig V.5 *The relation between I-ACTH and B-ACTH in the plasma of the normal subjects.*

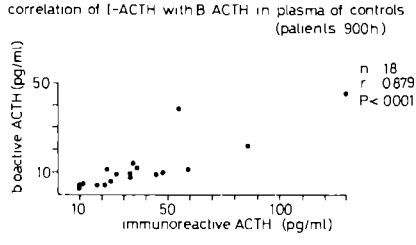

fig V.6 *The relation between I-ACTH and B-ACTH in the plasma of the patient control group.*

A statistically significant correlation was also found in the patients with untreated and treated Cushing's disease (fig. V.7 and V.8; r= 0.796, P <0.001 and r= 0.975, P <0.001 respectively). Fig V.7 further illustrates that only 7 out of 42 (16.7%) ACTH levels exceeded the normal range for both I-ACTH and B-ACTH, whereas 30 early morning values were within the normal range. Fig V.8 demonstrates in addition that substitution with corticosteroids obviously did not influence the relation between I-ACTH and B-ACTH values.

In the patients with Addison's disease a statistically significant correlation between I-ACTH and B-ACTH was found as well (fig V.9; r= 0.762, P <0.01).

Data on the relation between I-ACTH and B-ACTH are scarce. Fleisher et al (1974), using the cytochemical assay as a bioassay, reported a correlation coefficient of 0.94 in normal subjects between basal B-ACTH and I-ACTH. Liotta & Krieger (1975) using a multiple sampling procedure reported

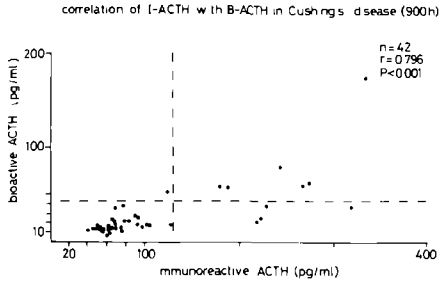

fig V.7 *Relation between I-ACTH and B-ACTH in the plasma of patients with untreated Cushing's disease. Upper normal values are presented by the underbroken lines.*

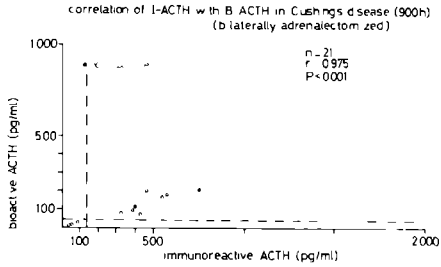

fig V.8 *Relation between I-ACTH and B-ACTH in the plasma of patients after bilateral adrenalectomy for Cushing's disease with and without substitution therapy.*

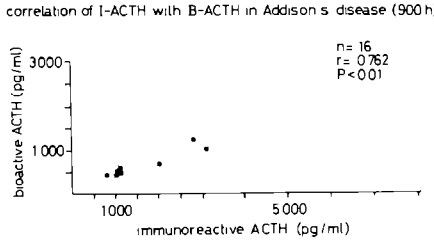

fig V.9 *Relation between I-ACTH and B-ACTH in the plasma of patients with Addison's disease. The open circle indicates the patient on substitution. x: values of the patient with a "Sertoli cell only" syndrome in addition to Addison's disease.*

correlation coefficients between 0.86 and 0.98 in two normal subjects, and in the same study mean values of 0.76 and 0.95 respectively in two patients with untreated Cushing's disease. Scrutinizing the data of Matsuyama et al (1972), who used the Lipscomb-Nelson assay as bioassay correlation coefficients ranging from 0.40 to 0.85 could be calculated from their data in patients bilaterally adrenalectomized for Cushing's disease, subjects treated with metyrapone, and in patients undergoing open heart surgery.

V.3.1.6 Relations between plasma I-ACTH or B-ACTH and plasma cortisol

Several considerations must be kept in mind when relating I-ACTH or B-ACTH with cortisol levels in plasma: the inherent biological variation in these relations, and more importantly so the facts that increases of ACTH levels precede increases of cortisol levels, and that the half-lives of cortisol (about 70 min; Peterson et al 1955) and ACTH (below 30 min; cfr review Bennett & McMartin 1979) differ greatly.

Table V.1 shows the correlation at 900 h a.m. between I-ACTH or B-ACTH and cortisol levels in the same plasma samples of control subjects and of patients with Cushing's disease. In the control group an albeit low but significant correlation ($r = 0.346$; $P < 0.05$) was found between I-ACTH and cortisol levels. Despite a tight relation between I-ACTH and B-ACTH (fig V.5 and V.6) such a statistically significant correlation between B-ACTH and plasma cortisol was not found in the control subjects ($r = 0.173$). In the patients with untreated Cushing's disease with their much higher ranges of ACTH and cortisol both I-ACTH and B-ACTH levels were significantly correlated with plasma cortisol levels ($r = 0.460$ for I-ACTH, and 0.451 for B-ACTH).

Table V.1

Correlation between I-ACTH or B-ACTH and cortisol at 900 h

	I-ACTH vs cortisol			B-ACTH vs cortisol		
	n	r	P^+	n	r	P^+
controls	40	0.346	<0.05	35	0.173	ns
patients with Cushing's disease	51	0.460	<0.005	36	0.451	<0.01

In fig V.10 and V.11 the relations between I-ACTH or B-ACTH and plasma cortisol in 29 patients with Cushing's disease are shown together with the upper normal ranges for both hormones. At 900 h 60% of both hormone levels were within the normal range for I-ACTH and cortisol (fig V.10). The percentages of values exceeding the normal range amounted to 22% for I-ACTH and to 31% for cortisol. Only in 14% of the values the levels of both hormones were elevated. Comparing B-ACTH and cortisol values in 36 plasma samples of patients with Cushing's disease, about 60% of both hormone levels fell within the normal range (fig V.11). The percentages of values exceeding the normal

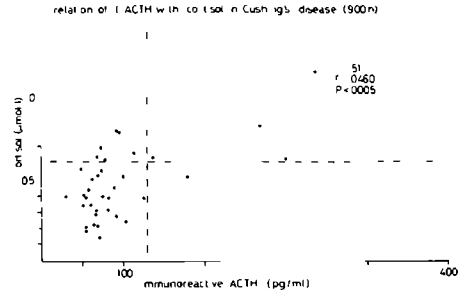

fig V.10 *The relation between I-ACTH and cortisol in plasma of patients with Cushing's disease. Broken lines indicate the upper normal values.*

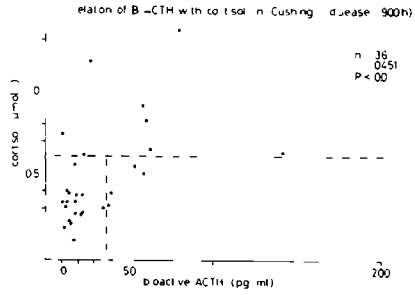

fig V.11 *The relation between B-ACTH and cortisol in plasma of patients with Cushing's disease. Broken lines indicate the upper normal values.*

range for each of the hormones amounted to 28%. These data (fig V.10 and V.11) illustrate that neither measurement of I-ACTH alone nor measurement of B-ACTH in the morning are diagnostic for the presence of Cushing's disease.

In the normal subjects of the present study a statistically significant correlation was found between plasma I-ACTH and cortisol but not between plasma B-ACTH and cortisol. This finding may be explained by the difference in half-life between B-ACTH and I-ACTH (Besser et al 1971b). As the half-life of I-ACTH is reportedly longer than that of B-ACTH, measurement of I-ACTH may reflect preceding ACTH stimulation to a greater extent than measurement of B-ACTH.

In contrast to the findings in normal subjects we did find a statistically significant correlation between B-ACTH and cortisol in plasma of patients with untreated Cushing's disease. A longer half-life of B-ACTH relative to I-ACTH in patients with Cushing's disease as compared to control subjects, a more tonic release of ACTH from the pituitary gland, a larger range of both B-ACTH and cortisol in these patients all might account for the statistically significant correlation that was found. However, there is no evidence that the decay of B-ACTH is lower in plasma of patients with untreated Cushing's disease than in control subjects. Furthermore, the release of cortisol and ACTH in Cushing's disease is not a tonic but an episodic event showing irregular oscillatory patterns (Krieger et al 1971 ; Krieger & Allen 1975). Therefore the most likely explanation of the finding of a statistically significant correlation between plasma B-ACTH and cortisol in patients with Cushing's disease is simply the wider range of both hormones.

V.3.2 Circadian rhythmicity of plasma Immunoreactive ACTH, Bioactive ACTH and cortisol

V.3.2.1 Control subjects

The diurnal changes in plasma I-ACTH, B-ACTH and cortisol during a 24 hours cycle were studied in 11 patients without clinical or biochemical evidence of pituitary-adrenal dysfunction (fig V.12). A circadian rhythmicity could be demonstrated for I-ACTH (P^{x} <0.05), B-ACTH (P^{x} <0.005) and cortisol P^{x} <0.005).

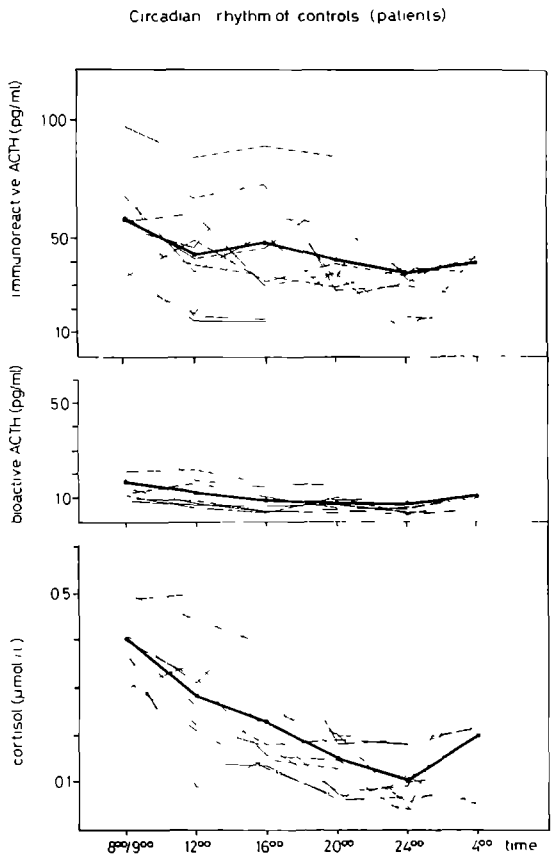

fig V.12 *Circadian rhythmicity of I-ACTH, B-ACTH and cortisol in plasma of control patients. Individual (————) as well as the mean values (▬▬▬) are presented.*

The mean I-ACTH levels (± SD) fell from 58.7 ± 21.4 at 800/900 h to 42.9 ± 21.8 at 1200 h, 47.9 ± 25.6 at 1600 h, 40.7 ± 20.3 at 2000 h, 35.1 ± 22.4 at 2400 h, and 35.7 ± 16.2 pg/ml at 400 h respectively. Only the mean I-ACTH values at 2000 h ($P^{xx}<0.05$), 2400 h ($P^{xx}<0.01$) and 400 h ($P^{xx}<0.02$) were significantly lower than the mean 800/900 h level. Although there was a tendency for the mean I-ACTH level at 1600 h to be lower than at 800/900 h, this difference lacked statistical significance ($0.05 < P^{xx} < 0.10$).

Plasma B-ACTH levels also showed circadian rhythmicity with mean levels of 16.7 ± 10.9 pg/ml at 800/900 h, 12.0 ± 6.2 at 1200 h, 9.0 ± 5.1 at 1600 h, 8.3 ± 3.8 at 2000 h, 7.7 ± 6.2 at 2400 h and 9.2 ± 4.8 pg/ml at 400 h respectively. The mean B-ACTH levels at 1600 h, 2000 h and 2400 h were all significantly lower than the 800/900 h levels ($P^{xx}<0.02$).

The mean I/B ratio ranged between 3.76 + 1.65 at 800/900 h to 5.10 ± 2.72 at 1600 h without overt circadian rhythm ($P^{x}>0.10$; fig V.13).

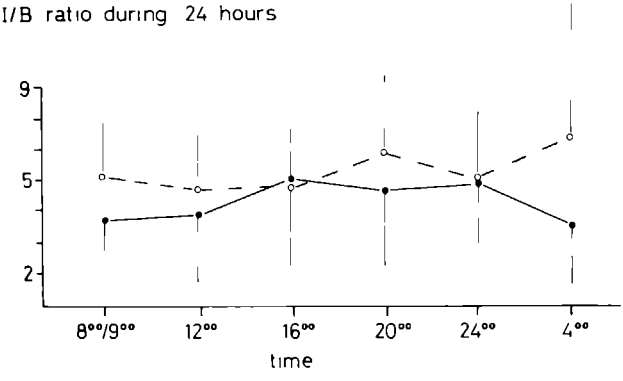

fig V.13 *The I/B ratio during a 24 hours cycle in control patients (●——● ; n=7) and in patients with Cushing's disease (o– – – o; n=9). The mean values are presented ± SD.*

Plasma cortisol levels in the control subjects showed the well-known circadian variation with an acme at 800/900 h and a nadir at 2400 h. At all time intervals the mean plasma cortisol values were significantly lower than at 800/900 h ($P^{xx}<0.01$).

It has to be emphasized that the diurnal rhythmicity of I-ACTH, B-ACTH and cortisol could be demonstrated despite the reported occurrence of episodic bursts of these hormones throughout the day (Hellman et al 1970; Weitzman et al 1971; Krieger et al 1971; Gallagher et al 1973; Krieger & Allen 1975).

V.3.2.2 Patients with Cushing's disease

In fig V.14 the individual and mean plasma I-ACTH, B-ACTH and cortisol levels at the time intervals mentioned above are shown and compared to the ranges in the control subjects.

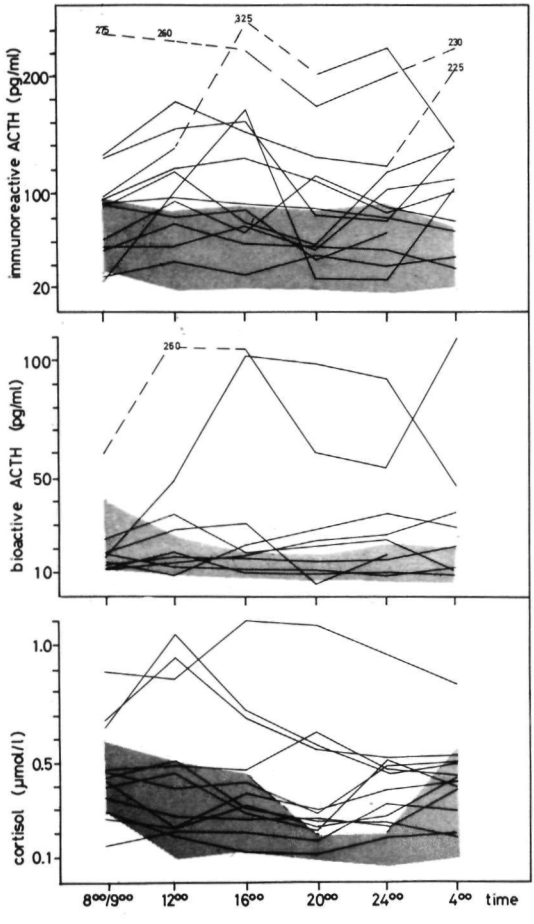

fig V.14 *Individual (————) levels of I-ACTH, B-ACTH and cortisol in plasma of patients with Cushing's disease. The shaded areas indicate the ranges of respective hormone levels in the control patient group.*

In contrast to the clearcut diurnal variation in control subjects no circadian rhythmicity for I-ACTH, B-ACTH or cortisol could be detected for this group of patients (P^{X} >0.10). No significant changes in the hormone values could be demonstrated in the time interval studied.

As in the control patient group the I/B ratio did not show a distinct rhythm ($P^{x}>0.10$). The mean I/B ratios ranged from a lowest value at 1200 h (4.73 $\pm$ 3.02) to a maximal value of 6.30 $\pm$ 4.35 at 400 h (fig V.13).

To assess the most appropriate time for discriminating between control subjects and patients with Cushing's disease, the percentages of hormone values exceeding the normal range were calculated at each time interval. The data compiled in table V.2 show that 800/900 h is the least appropriate time for discriminating. Measurement of cortisol in hospitalized patients at 2000 h and at 2400 h is the most discriminating.

Table V.2

Percentages of plasma I-ACTH, B-ACTH and cortisol levels in Cushing's disease exceeding the normal range at different times

	800/900 h	1200 h	1600 h	2000 h	2400 h	400 h
I-ACTH	38.5%	66.7%	61.5%	54.5%	76.9%	75 %
B-ACTH	11.1%	50.0%	77.8%	60.0%	55.6%	62.5%
cortisol	25.0%	33.3%	33.3%	83.3%	91.7%	16.7%

Calculating the mean of all plasma I-ACTH, B-ACTH and cortisol values at the different time intervals throughout the day, it appeared that I-ACTH values were elevated in 79%, B-ACTH values in 75%, and cortisol in 77% of the patients with Cushing's disease.

V.3.2.3 Patients with Addison's disease

Scarce data on the diurnal changes of I-ACTH and B-ACTH are shown in fig V.15. Similar to the profile in control subjects both I-ACTH and B-ACTH values were highest in the morning, decreased after that time and reached their nadir at midnight. At all time intervals both I-ACTH and B-ACTH levels were higher than in controls.
Due to the paucity of data meaningful calculations of the I/B ratio were not possible.

Krieger and Gewirtz (1974) using a frequent sampling technique - sampling took place every half hour - also demonstrated the presence of a diurnal variation of I-ACTH in plasma despite interfering episodic ACTH pulses. Other authors sampling blood at less frequent sampling times also found a nycthemeral ACTH variation (B-ACTH: Graber et al 1965; I-ACTH: Besser et al 1971; Oliver et al 1971) as in our study.

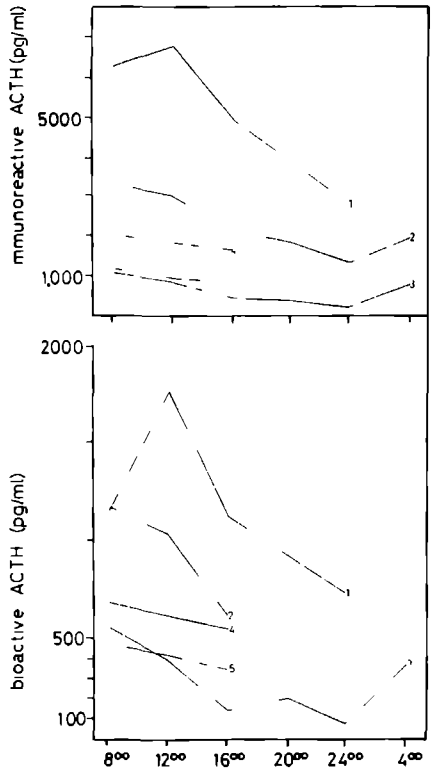

fig V.15 *Circadian variation in patients with Addison's disease. Numbers indicate the I-ACTH and B-ACTH values obtained from the same patient. Patient 1 had a "Sertoli cell only" syndrome in addition.*

V.3.3 Circadian variation of plasma Immunoreactive ACTH, Bioactive ACTH and cortisol between 900 h and 1600 h

For reasons of convenience circadian variation was additionally studied by measuring ACTH and cortisol in the morning and afternoon. It is noted that in the control patient group 11 out of 15 data and in the groups of patients with Cushing disease and Addison's disease all data were taken from rhythm studies, whereas in the laboratory control group casual samples were studied.

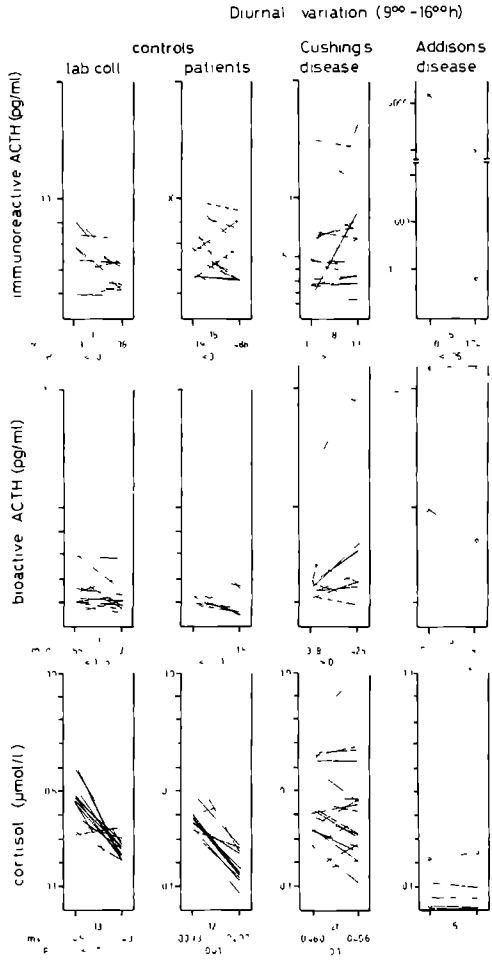

fig V.16 *Variations in plasma I-ACTH, B-ACTH and cortisol between 900 h and 1600 h. Open circles point to the patient with substitution, crosses point to the patient with a "Sertoli cell only" syndrome in addition to Addison's disease.*

V.3.3.1 I-ACTH

In fig V.16 the individual plasma I-ACTH, B-ACTH and cortisol levels at 900 h and at 1600 h in control subjects, in patients with untreated Cushing's disease and in patients with Addison's disease are given.

In both control groups the mean I-ACTH values decreased significantly between 900 h and 1600 h (from 55.3 $\pm$ 30.5 pg/ml to 38.3 $\pm$ 17.4 pg/ml for the

laboratory collaborators, P^{xx} <0.05; from 63.9 ± 28.4 pg/ml to 48.6 ± 23.6 pg/ml for the control patients, P^{xx} <0.05). In 11 out of 13 (85%) laboratory collaborators and in 13 out of 15 subjects (87%) in the control patient group plasma I-ACTH levels were lower at 1600 h than at 900 h. If we consider only the data derived from the rhythm study in 9 out of 11 (82%) patients I-ACTH levels proved to be lower than at 900 h.

In the hospitalized patients with untreated Cushing's disease the mean I-ACTH value did not change significantly between 900 h and 1600 h. The 1600 h values in these patients ranged from 31 to 325 pg/ml. As is shown in fig V.17 the 1600 h values were lower in only 8 out of 18 patients (44%).

In the patients with Addison's disease I-ACTH levels were invariably lower at 1600 h than at 900 h (P^{xx} <0.05) with a range varying from 320 to 5000 pg/ml at 1600 h. The mean relative decrease (28%) in Addison's disease throughout the day was similar to that in both control groups (31% for the laboratory collaborators; 24% for the control patients).

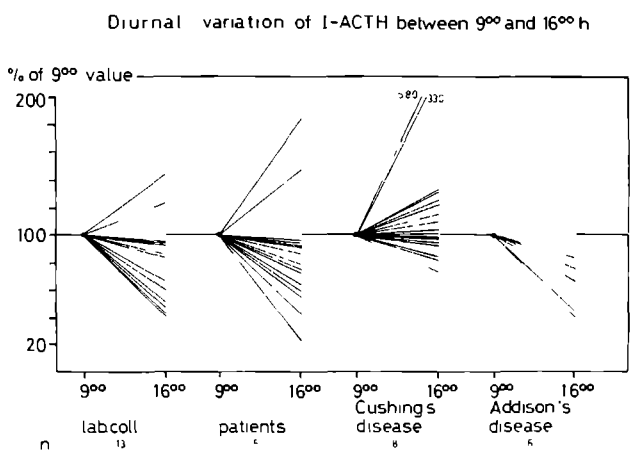

fig V.17 *Relative variation of I-ACTH in plasma between 900 h and 1600 h.*

V.3.3.2 B-ACTH

In both control groups the B-ACTH levels also decreased significantly between 900 h and 1600 h from a mean value of 16.5 ± 9.5 pg/ml to 13.5 ± 7.8 pg/ml (P^{xx} <0.05) in the laboratory collaborators and from 19.2 ± 13.7 pg/ml to 11.6 ± 9.9 pg/ml (P^{xx} <0.01) in the patient control group. The 1600 h values in the control subjects ranged from 4.5 to 35 pg/ml (fig V.16). The relative

changes in B-ACTH values in all groups of control subjects and patients are given in fig V.18. In 10 out of 13 (77%) non-hospitalized laboratory collaborators and 9 out of 9 control patients B-ACTH values at 1600 h were lower than at 900 h.

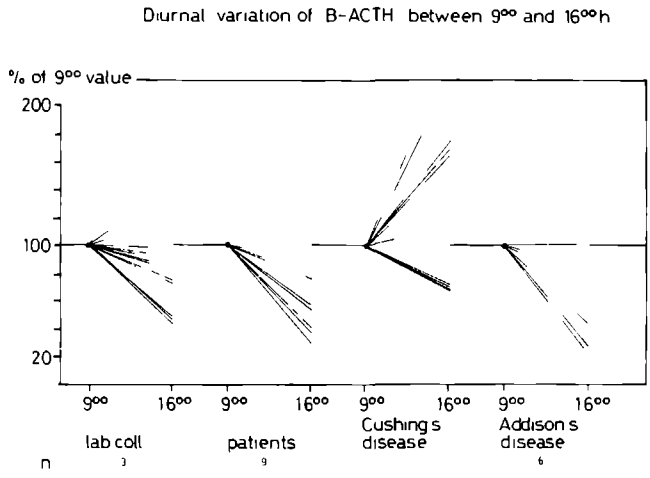

fig V.18 *Relative variation of B-ACTH in plasma between 900 h and 1600 h.*

In the hospitalized patients with untreated Cushing's disease the mean B-values at 1600 h (42.5 ± 38.4 pg/ml) did not differ significantly from the 900 h value (31.8 ± 38.6 pg/ml, P^{xx} >0.10). The 1600 h values in these patients ranged from 9 to 105 pg/ml. In the patients with untreated Cushing's disease only 4 out of 11 B-ACTH values (36%) were lower at 1600 h than at 900 h.

In all but one of the patients with Addison's disease a diurnal decrease occurred. Only in the patient with Addison's disease in combination with the "Sertoli cell only" syndrome the B-ACTH value remained virtually unchanged.

In none of the groups of the control subjects, the patients with Cushing's disease or Addison's disease, a statistically significant change in I/B ratio was found between 900 h and 1600 h (P^{xx} >0.10; table V.3).

Table V.3

I/B ratio's at 900 h and 1600 h

	n	900 h	1600 h	P^{xx}
laboratory coll.	13	3.50 ± 1.16	3.02 ± 0.73	>0.10
control patients	9	3.98 ± 1.38	4.88 ± 2.58	>0.10
patients with Cush.Dis.	11	5.77 ± 2.73	4.77 ± 2.73	>0.10
patients with Add.Dis.	6	2.85 ± 1.38	2.96 ± 0.89	>0.10

V.3.3.3 Cortisol

In both control groups plasma cortisol significantly decreased between 900 h and 1600 h (P^{xx} <0.01). The relative changes of plasma cortisol between 900 h and 1600 h are shown in fig V.19. In 12 out of 13 (92%) laboratory collaborators as well as in 12 out of 13 patient controls (92%) cortisol values at 1600 h were lower than the 900 h values.

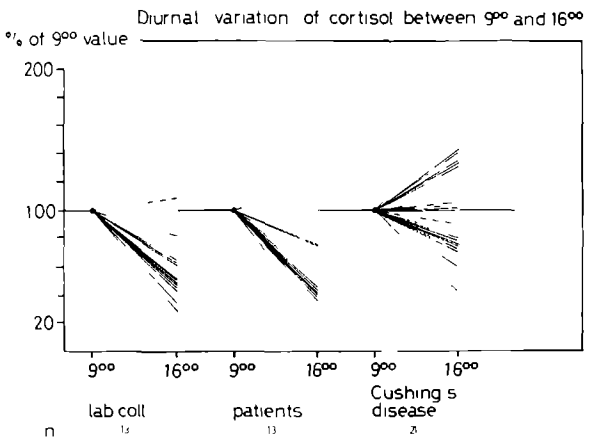

fig V.19 *Relative variation of cortisol in plasma between 900 h and 1600 h.*

In the patients with untreated Cushing's disease the mean cortisol value at 1600 h (0.460 ± 0.188 µmol/l) did not differ significantly from the 900 h value (0.456 ± 0.239 µmol/l, P^{xx} >0.10). As is shown in fig V.19 in only 10 out of 21 patients the 1600 h values were lower. If we take a decrease of at least 25% (Krieger et al 1971) between 900 h and 1600 h as a criterium, 22 out of 26 (85%) control subjects of the present study fulfilled this criterium. This percentage is similar to that reported by Krieger et al (1971) who found 22% of the plasma cortisol levels to be less than their morning value. In Cushing's disease 4 out of 21 values (19%) were below this 25% criterium, as was confirmed in the present study.

In fig V.20 the relation between plasma cortisol and both I-ACTH and B-ACTH at 1600 h in patients with untreated Cushing's disease together with the upper control values are depicted. If we compare the I-ACTH and cortisol levels it appears that about 35% of the values are within the normal range. The 900 h values of the same subjects on the same day show that the percentage of hormone levels falling within the normal range of 900 h was as high as about 60% (cfr

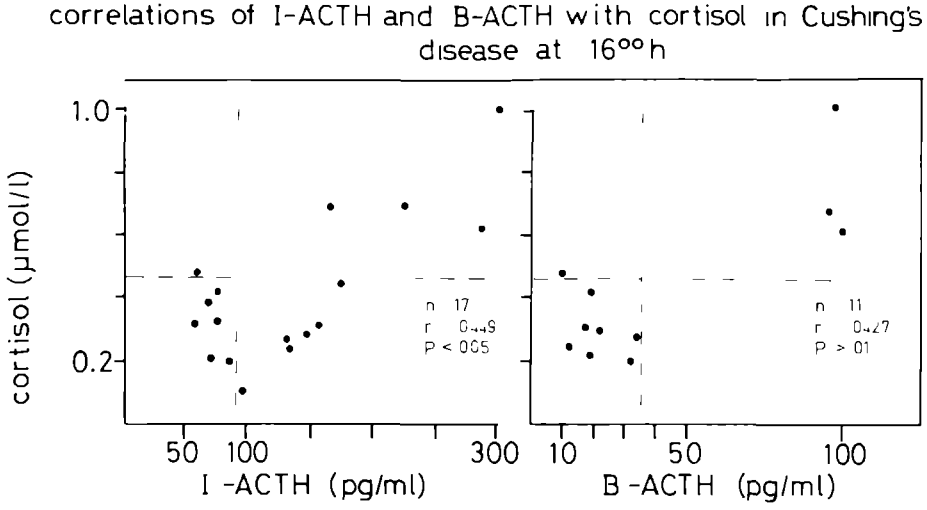

fig V.20 *The relations of I-ACTH and B-ACTH with cortisol in plasma of patients with Cushing's disease. The broken lines point to the upper values of I-ACTH, B-ACTH and cortisol in plasma of the control subjects at 1600 h.*

fig V.10). If we compare the B-ACTH and cortisol values in the same way the percentage of hormone levels overlapping the normal range appears to be 63% for 1600 h and 73% for 900 h. Therefore, the absolute cortisol and I-ACTH or B-ACTH values even at 1600 h do not discriminate completely between euadrenocorticism and hyperadrenocorticism.

V.3.3.4 Comparison with literature

In spite of the occurrence of episodic bursts of both ACTH and cortisol secretion (Hellman et al 1970; Krieger et al 1971), casual measurement of these hormones yet can give further information about the pituitary-adrenal gland function. Already in 1963 Ney et al using the Lipscomb-Nelson bioassay, found that in 10 out of 12 normal subjects the mean 1800 h ACTH values of the group were lower than at 600 h. Using radioimmunoassays a similar diurnal decrease of ACTH was observed by several other authors (Demura et al 1966; Besser & Landon 1968; Berson & Yalow 1968; Matsukara et al 1971; Ichikawa et al 1971). Wolfsen et al (1972) - using a radioreceptor assay - also found lower ACTH levels in the afternoon. The present study also showed a statistically significant decrease of I-ACTH, B-ACTH and cortisol between 900 h and 1600 h.

In patients with Cushing's disease no circadian variation exists (Doe et al 1960). Therefore, in these patients the 1600 h plasma ACTH values are also expected to occur at random above or below the 900 h values. Summarizing the data of fig V.17, V.18 and V.19, we could state that about half of all 1600 h I-ACTH, B-ACTH and cortisol values were below the 900 h values in patients with Cushing's disease, whereas in the total control group about 90% of all 1600 h values were below the 900 h values. It should be noted again that in these studies most of the 900 h and 1600 h values of patients were taken from series of diurnal variations.

Our observations about rhythmicity of I-ACTH and B-ACTH in patients with Addison's disease reconfirm data of Graber et al (1965), who used the Lipscomb-Nelson assay, and of Besser et al (1971), who used a radioimmunoassay.

V.3.4 Short-term variation in plasma Immunoreactive ACTH, Bioactive ACTH and cortisol between 900 h and 930 h.

In order to study the effect of repeated venous puncture in a short time interval two blood samples were taken; the first at 900 h, the second at 930 h in a group of collaborators of the laboratory, in the patient control group and in patients with Cushing's disease.

V.3.4.1 Short-term variation in plasma I-ACTH levels.

The changes in plasma I-ACTH values between 900 h and 930 h are depicted in fig V.21. In both control groups I-ACTH levels decreased significantly from 54.5 $\pm$ 35.4 pg/ml at 900 h to 39.9 $\pm$ 26.1 pg/ml at 930 h for the laboratory

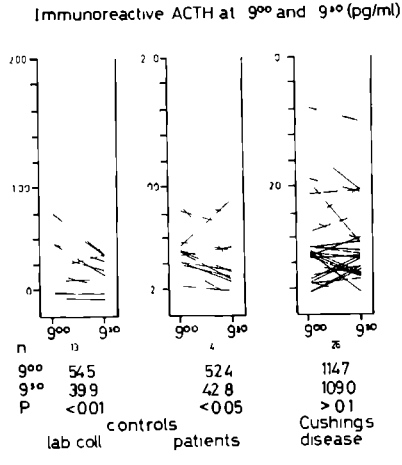

fig V.21 *Short-term variation of I-ACTH in human plasma between 900 h and 930 h.*

collaborators (P^{xx} <0.01), and from 52.4 ± 19.1 pg/ml to 42.8 ± 21.8 pg/ml for the patient control group (P^{xx} <0.05). In fact, 21 out of 27 I-ACTH values (78%) in the total control group were lower at 930 h than at 900 h.

In contrast, in the group of patients with Cushing's disease the plasma I-ACTH levels remained virtually unchanged (114.7 ± 70.8 pg/ml at 900 h and 109.0 ± 62.0 pg/ml at 930 h; P^{xx} >0.10). Fourteen out of 26 samples (54%) showed lower levels at 930 h than at 900 h.

V.3.4.2 Short-term variation in plasma B-ACTH levels.

The B-ACTH values are shown in fig V.22. In the laboratory collaborators B-ACTH values decreased from 16.1 ± 8.7 pg/ml at 900 h to 12.5 ± 6.8 pg/ml at 930 h (P^{xx} <0.05). In the patient control group B-ACTH levels also decreased: from 15.9 ± 9.1 pg/ml at 900 h to the lower value of 11.5 ± 6.2 pg/ml at 930 h (P^{xx} <0.05). In fact, 20 out of 25 (80%) B-ACTH levels in the total control group were lower at 930 h than at 900 h.

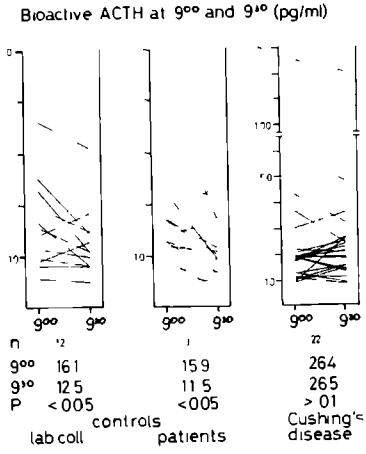

fig V.22 *Short-term variation of B-ACTH in human plasma between 900 h and 930 h.*

The I/B ratio in the whole control group virtually did not change between the two sampling times (3.62 ± 1.19 at 900 h versus 3.64 ± 1.62 at 930 h).

In the patients with Cushing's disease the mean B-ACTH levels were almost identical at 900 h and at 930 h (values: 26.4 ± 30.0 pg/ml and 26.5 ± 27.2 pg/ml respectively). In 10 samples B-ACTH levels were lower at 930 h than at 900 h.

As in the control group, no statistically significant change in I/B ratio was found between 900 h (5.45 ± 2.97) and 930 h (5.71 ± 2.97) in the patients with Cushing's disease.

V.3.4.3 Short-term variation in plasma cortisol levels

The changes in plasma cortisol levels between 900 h and 930 h are given in fig V.23. The mean plasma cortisol level in the laboratory collaborators decreased from 0.457 ± 0.102 µmol/l at 900 h to 0.402 ± 0.096 µmol/l at 930 h (P^{xx} <0.01). In the control patient group plasma cortisol significantly decreased as well, from 0.352 ± 0.143 µmol/l at 900 h to 0.279 ± 0.118 µmol/l at 930 h (P^{xx} <0.01). Only on two occasions the plasma cortisol values were higher at 930 h than at 900 h (8%).

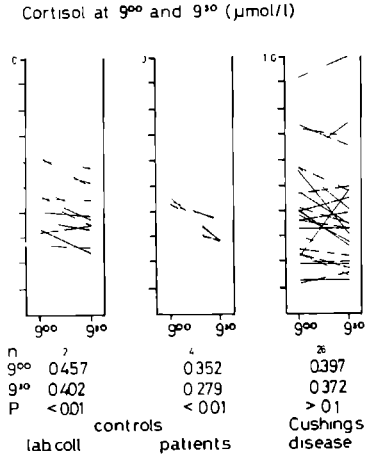

fig V.23 *Short-term variation of cortisol in human plasma between 900 h and 930 h.*

In the patients with Cushing's disease the plasma cortisol levels were similar at 900 h (0.372 ± 0.212 µmol/l) and at 930 h (0.397 ± 0.202 µmol/l; P^{xx} >0.10). In these patients 14 out of 26 cortisol levels were lower at 930 h than at 900 h.

V.3.4.4 Comparison of the short-term variation between 900 h and 930 h and the circadian variation between 900 h and 1600 h of hormone levels

In tables V.4 and V.5 data are given about the number of control subjects and patients with Cushing's disease showing lower hormone levels at 1600 h and 930 h than at 900 h.

It is remarkable that the number of subjects and patients showing a decrease between 900 h and 930 h is almost similar to that between 900 h and 1600 h. In the control subjects about 80% of the 1600 h and 930 h values for I-ACTH and B-ACTH were below the 900 h value and 90% of the plasma cortisol levels. In the patients with Cushing's disease about 50% of the I-ACTH, B-ACTH

Table V.4

Percentages and numbers of plasma samples with hormone levels at 1600 h below the 900 h levels

	control groups		patients with
	lab.coll.[x]	patients[xx]	Cushing's disease
I-ACTH	11/13= 84.6%	9/11= 81.8%	8/18= 44.4%[a]
B-ACTH	10/13= 76.9%	7/7 = 100.0%	4/11= 36.4%[b]
cortisol	12/13= 92.3%	10/11= 90.9%	10/21= 47.6%[c]

[x] casual blood sampling

[xx] blood samples obtained from the circadian rhythm study

[a] p^+ >0.10 versus the control groups

[b] p^+ <0.025 versus the patient control group and >0.10 versus the lab. coll.

[c] p^+ <0.025 versus the lab.coll. group and <0.05 versus the patient control group

Table V.5

Percentages and numbers of plasma samples with hormone levels at 930 h below the 900 h levels

	control groups		patients with
	lab. coll.	patients	Cushing's disease
I-ACTH	9/13= 69.2%	12/14= 85.7%	14/26= 53.8%[a]
B-ACTH	9/12= 75.0%	11/13= 84.6%	10/22= 45.5%[a]
cortisol	11/12= 91.7%	13/14= 92.9%	14/26= 53.8%[b]

[a] p^+ >0.10 versus control groups

[b] p^+ <0.05 versus patient control group and >0.10 versus the lab.coll.

and cortisol levels at 1600 h and 930 h were below the 900 h values. For this similarity of the 930 h and 1600 h values two reasons might be considered: first, the diurnal variation per se, starting in the early morning and, secondly, the influence of adaptation to repeated venous puncture. As is well known from other stimuli of the hypothalamic-hypophyseal-adrenal system, in pituitary-dependent Cushing's disease an intrinsic extinction of such stimuli as mentioned above could be expected.

Surveying these data we might conclude that sampling of blood at 900 h and 930 h could be valuable in evaluating the pituitary-adrenal gland function.

V.3.5 Dexamethasone suppression test

V.3.5.1 I-ACTH

The results of the suppression of I-ACTH by dexamethasone are shown in fig V.24. In 9 out of 10 control subjects I-ACTH precipitously decreased overnight to an undetectable value (<10 pg/ml) after administration of 1 respectively 2 mg of dexamethasone at 2300 h the late evening before.

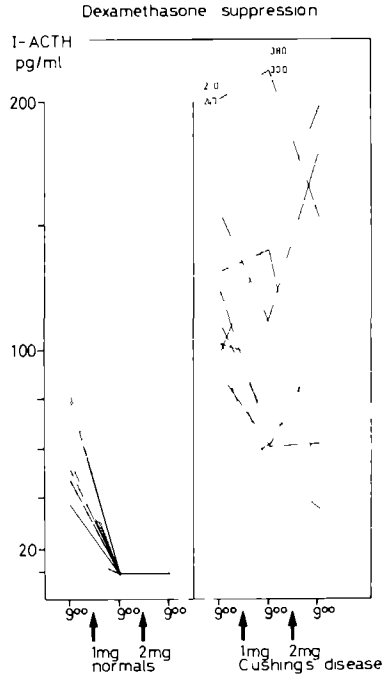

Fig V.24 I-ACTH plasma levels in the single dose overnight dexamethasone suppression test. Limit of detection about 10 pg/ml.

In the patients with pituitary-dependent Cushing's disease the mean plasma I-ACTH level virtually remained unchanged: 131.1 ± 63.3 pg/ml before dexamethasone treatment, 125.5 ± 111.9 pg/ml after 1 mg, and 106.0 ± 63.4 pg/ml after 2 mg of dexamethasone (P >0.10). In all patients plasma I-ACTH levels both after 1 and 2 mg dexamethasone were higher than in the control subjects. Together these data confirm the well-known loss of appropriate feedback in Cushing's disease.

The present findings augment data from literature on 1 or 2 mg single dose dexamethasone suppression of I-ACTH. In our study all but 1 control subjects showed a virtually complete suppression of I-ACTH after 1 mg dexamethasone, whereas post-treatment I-ACTH levels in control subjects were invariably lower

than in any of the patients with Cushing's disease. It should be emphasized, however, that lack of complete suppression of I-ACTH does not seem pathognomonic for the diagnosis of Cushing's disease. Landon & Greenwood (1968) administered 2 mg of dexamethasone the night before and found a detectable I-ACTH in 1 out of 14 control subjects, whereas Krieger et al (1979) reported detectable values in 3 out of 6 normal subjects after 1 mg of dexamethasone.

V.3.5.2 B-ACTH

To our knowledge no reports exist about B-ACTH in normal subjects after single dose overnight dexamethasone administration. As was noted earlier for I-ACTH, overnight administration of 1 mg respectively 2 mg dexamethasone decreased B-ACTH to undetectable B-ACTH values in 9 out of 10 control subjects (fig V.25). In one control subject both I-ACTH and B-ACTH remained detectable.

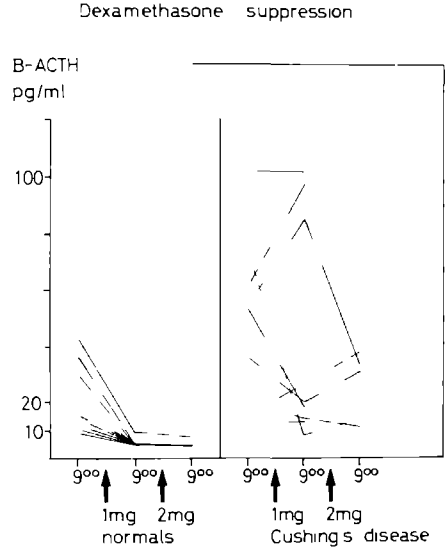

fig V.25 *B-ACTH plasma levels in the single dose overnight dexamethasone suppression test. Limit of detection about 4 pg/ml.*

In the patients with Cushing's disease the mean B-ACTH level was 47.4 ± 29.8 pg/ml before dexamethasone treatment, 44.5 ± 42.2 pg/ml after 1 mg, and 24.8 ± 11.9 pg/ml after 2 mg. In all patients with Cushing's disease B-ACTH levels both after 1 and 2 mg dexamethasone were higher than in the control subjects.

The mean I/B ratio in patients with Cushing's disease virtually remained unchanged before and after 1 or 2 mg dexamethasone (4.09 ± 2.10 before treatment, 4.14 ± 2.14 after 1 mg, and 4.97 ± 0.93 after 2 mg; P >0.10 between all values).

V.3.5.3 Cortisol

The effect of dexamethasone administration on plasma cortisol is shown in fig V.26. All plasma cortisol values were below 0.07 µmol/l after administration of 1 mg dexamethasone overnight. Administration of 2 mg of dexamethasone did not decrease the plasma cortisol level any further.

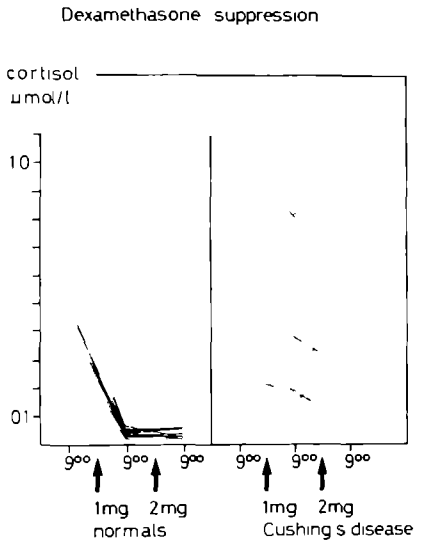

fig V.26 Cortisol plasma levels in the single dose overnight dexamethasone suppression test.

In the patients with Cushing's disease cortisol levels after 1 mg dexamethasone were invariably higher than 0.1 µmol/l (mean cortisol level 0.572 ± 0.232 µmol/l before treatment, and 0.430 ± 0.300 µmol/l after 1 mg; P >0.10). After administration of 2 mg the mean plasma cortisol level did not change significantly either (0.384 ± 0.287 µmol/l; P >0.10 versus basal level). In three patients plasma cortisol levels after two mg dexamethasone were higher than before treatment, whereas in two of these patients the 2 mg dexamethasone cortisol level exceeded the 1 mg dexamethasone value. It should be emphasized that in 1 patient with Cushing's disease plasma cortisol was below the arbitrarily chosen value of 0.1 µmol/l. In this patient, however, the suppression of I-ACTH and B-ACTH was not complete.

I-ACTH AND B-ACTH RESPONSE TO INSULIN-INDUCED HYPOGLYCAEMIA

VI.1 Introduction

It is generally accepted that plasma ACTH levels measured by radioimmunoassay are higher than those measured by bioassay (chapter IV). In this chapter the relation of I-ACTH to B-ACTH will be described and discussed in situations in which endogenous ACTH levels change rapidly in control subjects as a consequence of insulin-induced hypoglycaemia.

The insulin tolerance test has been in use for as long as 40 years as a test of hypothalamo-pituitary-adrenal function (Fraser et al 1941), an insulin-induced hypoglycaemia being followed by an increase of plasma cortisol after a preceding rise in plasma I-ACTH (Berson & Yalow 1968). Data on B-ACTH during insulin-induced hypoglycaemia are scarce in spite of the availability of very sensitive bioassays (isolated adrenal cell assays, Sayers et al 1971a; Lowry et al 1973; and cytochemical assays, Chayen et al 1972). Fleisher et al (1974), using both a radioimmunoassay and a cytochemical bioassay, found that after insulin-induced hypoglycaemia the mean I-ACTH to B-ACTH ratio further increased. After reaching the highest ACTH concentration at 60 minutes this ratio remained virtually unchanged during the next 30 minutes. On the other hand Liotta & Krieger (1975) - using the isolated rat adrenal cell system as a bioassay - showed preliminary data suggesting a further increase of the I/B ratio between 30 and 90 minutes after reaching both the I-ACTH and B-ACTH peak.

This study demonstrates the occurrence of a biphasic response in I/B ratio of endogenous ACTH in normal subjects during insulin-induced hypoglycaemia: the expected decrease of B-ACTH relative to I-ACTH was preceded by a temporary increase.

VI.2 Materials and Methods

Endogenous ACTH stimulation as induced by insulin-induced hypoglycaemia in control subjects

In twelve women without pituitary-adrenal gland dysfunction, hypoglycaemia was induced by intravenous injection of 0.1 U insulin/kg body weight. The

hypoglycaemia stimulus was considered adequate if the plasma glucose levels fell below 2 mmol/l. In all subjects the test was started between 900 and 1000 h. Blood samples were drawn twice before (-15 and 0 minutes) and at 20, 30, 45, 60 and 90 minutes after insulin injection. Blood was collected and plasma obtained as described before. In 7 other women without pituitary-adrenal gland axis dysfunction blood samples were drawn in the same period of the day at only time zero (between 900 and 1000 h) and at 20, 30 and 60 minutes after injection of physiological saline.

Assays

ACTH and cortisol assays were performed as described above. Blood glucose was measured by Autoanalyzer.

Statistics

Statistical analysis was performed by using Friedman's non-parametric analysis of variance (P), Wilcoxon's signed rank test (P^x), and Spearman's rank correlation test (P^+). Correlation coefficients are denoted by r. Unless otherwise stated the mean values were given $\pm$ SD. All samples from one subject were measured in the same assay.

Half-lives

Rough estimates of biological and immunological half-life of ACTH were made by plotting plasma ACTH against time and constructing a straight line to fit the points (Matsuyama et al 1972a).

VI.3 Results and Discussion

The effect of insulin-induced hypoglycaemia on plasma I-ACTH, B-ACTH and cortisol levels and on the I/B ratio are summarized in table VI.1 and fig VI.1. No statistically significant difference ($P^x > 0.10$) has been found between the mean plasma ACTH levels at -15 and 0 minutes, neither in the radioimmunoassay (t= -15: 21.3 $\pm$ 6.5 pg/ml and t= 0: 23.6 $\pm$ 11.0 pg/ml), nor in the bioassay (t= -15: 6.7 $\pm$ 2.8 pg/ml and t= 0: 7.1 $\pm$ 3.4 pg/ml). In each subject a distinct increase in both I-ACTH and B-ACTH levels was observed 30 minutes after the insulin injection i.e. 10 minutes after the onset of hypoglycaemia (I-ACTH: 104.3 $\pm$ 80.5 pg/ml, $P^x < 0.001$ versus t=0 and B-ACTH: 62.3 $\pm$ 66.6 pg/ml, $P^x < 0.001$ versus t=0). After that the mean I-ACTH and B-ACTH plateaued until

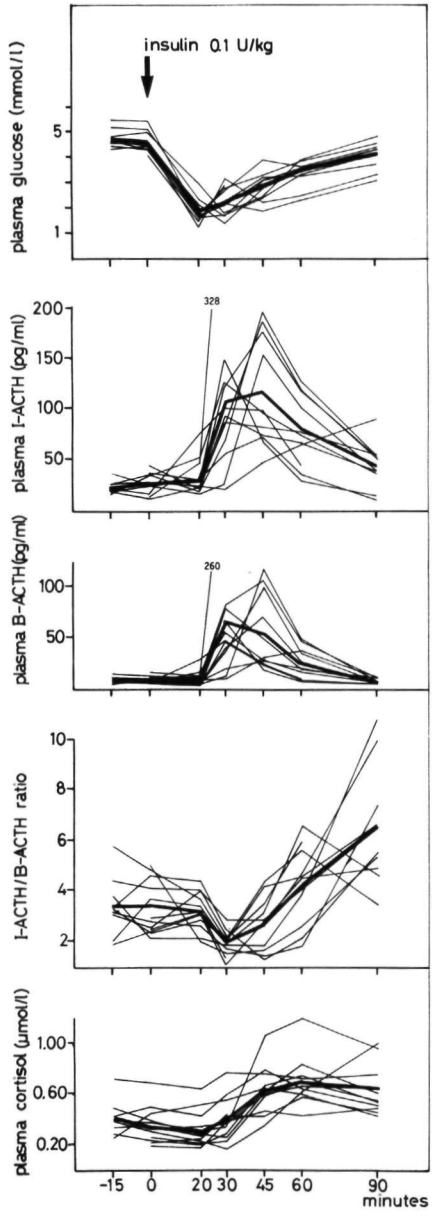

fig VI.1 *Individual (————) and mean (━━━━) response of plasma glucose, I-ACTH, B-ACTH and cortisol and of the I/B ratio in control subjects upon insulin administration.*

Table VI.1

Simultaneous plasma hormone profiles during insulin-induced hypoglycaemia in an example subject

	time (min)						
	-15	0	20	30	45	60	90
I-ACTH (pg/ml)	19	23	16	67	195	128	55
B-ACTH (pg/ml)	5	10.5	5	34	99	33	5
I/B ratio	3.80	2.19	3.20	1.97	1.97	3.88	11.00
cortisol (μmol/l)	0.39	0.38	0.32	0.34	1.07	1.26	0.96

45 minutes and then gradually fell. The I-ACTH levels remained significantly elevated until the end of the test (t=90: 43.7 ± 22.2 pg/ml versus t-0, P^{x} <0.05), whereas the bioactive levels returned to baseline values at t=90 minutes (6.8 ± 3.2 pg/ml versus t=0, P^{x} >0.10). Table VI.2 shows that at most sampling times the individual I-ACTH and B-ACTH levels were significantly and tightly correlated.

The mean I/B ratios at -15 and 0 minutes did not differ significantly (3.43 ± 1.19 versus 3.45 ± 1.08, P^{x} >0.10). Insulin-induced hypoglycaemia elicited a biphasic response in the I/B ratio (P <0.001): initially the mean ratio decreased from 3.45 ± 1.08 at t=0 minutes to a lowest value of 1.98 ± 0.51 at 30 minutes (P^{x} <0.01). The 30 minutes value was also significantly lower than the 20 minutes value (3.32 ± 0.66, P^{x} <0.01). After that the mean ratio gradually rose (P <0.005) from 1.98 ± 0.51 at 30 minutes to 2.69 ± 1.08 at t=45 minutes, 4.19 ± 1.57 at 60 minutes (P^{x} <0.01 vs 30 minutes) and 6.54 ± 2.55 at 90 minutes (P^{x} <0.01 vs 30 minutes). Only the 90 minutes value was significantly higher than the value at time zero (P^{x} <0.01).

The effects of injection of physiological saline in control subjects on plasma I-ACTH,B-ACTH and cortisol as well as on the I/B ratio are shown in fig VI.2. The cortisol levels showed the downward trend as presumably caused by the diurnal rhythmicity (P <0.005). The I-ACTH and B-ACTH levels did not change significantly during the test nor did the mean I/B ratio (I/B ratio at t=0: 3.22 ± 1.28; t=20: 3.45 ± 0.70; t=30: 3.39 ± 1.20 and t=60: 3.20 ± 0.79; P >0.10).

As discussed earlier (chapter IV & V) the bioactive ACTH values in the subjects studied are lower than the immunologically assayed ACTH values under basal circumstances. The hypoglycaemic stimulus immediately induced a release of ACTH. At the same time a 40% decrease of the I/B ratio from 3.45 at t=0 to

Table VI.2

Correlations of plasma I-ACTH, B-ACTH and cortisol during insulin induced hypoglycaemia

		I-ACTH vs B-ACTH		I-ACTH vs cortisol		B-ACTH vs cortisol	
time	n	r	p^+	r	p^+	r	p^+
-15	9	0.442	ns	0.504	ns	0.225	ns
0	12	0.823	<0.005	-0.273	ns	-0.132	ns
20	12	0.816	<0.005	-0.461	ns	-0.593	<0.05
30	11	0.909	<0.001	0.460	ns	0.477	ns
45	11	0.664	<0.025	0.209	ns	-0 176	ns
60	11	0.721	<0.025	0 755	<0.01	0.464	ns
90	10	0.700	<0.025	0.873	<0.001	0 600	<0.05

1.98 at t=30 minutes was observed which was transient and was followed by an increase of this ratio far exceeding the pre-treatment value (fig VI.1). After reaching a plateau the mean B-ACTH level rapidly declined - with a roughly estimated half-life of 11.6 $\pm$ 1.8 min - so that at t=90 minutes the B-ACTH level did not differ further from the baseline value. But in contrast to B-ACTH, I-ACTH disappeared more slowly - half-life being 19.6 $\pm$ 5.6 min - so that the mean I-ACTH level at 90 minutes was still higher than the pre-treatment value. The most plausible explanation for this difference in disappearance rates for B-ACTH and I-ACTH is that at the decay phase ACTH metabolites arise which are still immunoassayable but not bioassayable.

Only two reports exist dealing with the effect of insulin-induced hypoglycaemia on the I-ACTH, and B-ACTH levels in plasma. Fleisher et al (1974) observed a rise of the I/B ratio 60 minutes after the insulin injection, but did not find a decrease of the I/B ratio. It should be noted that these authors used the cytochemical assay as a bioassay and collected blood at half hour intervals. Liotta & Krieger (1975), using isolated adrenal cells for bioassay, did not find a significant decrease either of the I/B ratio in an admittedly small group of subjects.

In view of the time course required for ACTH to stimulate cortisol production and in view of the differences of the half-lives of both compounds, the quantitative relation between B-ACTH and I-ACTH on one hand and cortisol on the other hand was expectedly poor, confirming data of Daly et al (1973) and Krieger & Allen (1975).

Analogous to the hypoglycaemia-induced ACTH release in normal subjects

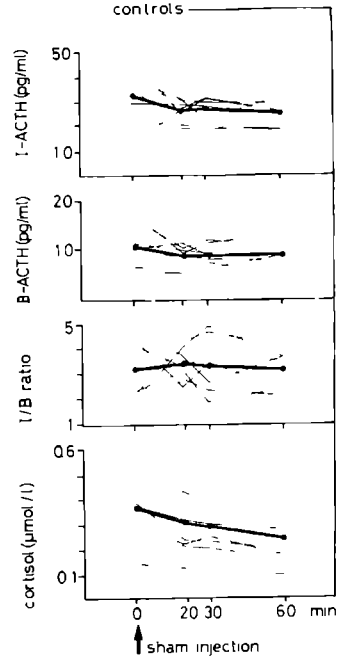

fig VI.2 *The effect of injection of physiological saline upon the individual (————) and mean (━━━━) response of plasma I-ACTH, B-ACTH and cortisol and of the I/B ratio in control subjects.*

another experiment was performed in our laboratory in a sub-group of patients with Cushing's disease, who evinced a paradoxical rise of plasma cortisol (Pieters et al 1979) as well as ACTH (Pieters et al, in preparation) after a non-specific stimulus such as TRH and/or LHRH. Remarkably a similar transient decrease of the I/B ratio was observed in these patients. It is striking that in this condition release of B-ACTH and I-ACTH from the pituitary gland was also accompanied by a transient decrease of the I/B ratio again by about 40% (from 3.27 $\pm$ 0.58 at t=0 minutes to 1.85 $\pm$ 0.66 at t=10 minutes after the ACTH stimulus).

Together the data in the ACTH stimulation tests in normal subjects and in patients with Cushing's disease indicate that under basal circumstances ACTH levels determined by radioimmunoassay overestimated the levels measured by bioassay.

SUMMARY

In this thesis the development of a biological as well as of a radioimmunochemical method for the determination of ACTH in human blood has been described. The study included the comparison of biological and immunological ACTH concentrations in blood under different physiological, pathological and experimental conditions.

The extensive literature data concerning characteristics of ACTH and its regulation were briefly surveyed in the general introduction (chapter I). The aim of the study and the reason for choosing the isolated adrenal cell system as a bioassay have also been explained in this chapter.

In chapter II the development and the evaluation of the bioassay has been described. Cells were isolated from the adrenal tissue by collagenase disruption and suspended in buffer. In order to obtain an as sensitive assay as possible several modifications in the original assay - as reported in literature - were applied and evaluated. The most important improvements, which have resulted in a gain in sensitivity, were the purification of the isolated adrenal cells - by passage through a 5% BSA layer - followed by a pre-incubation of these cells. The gain in sensitivity was not the same for all ACTH peptides tested. After purification and pre-incubation of the cells, which involves presumably a removal and/or deactivation of ACTH-attacking enzymes, it appeared that the gain in sensitivity of the assay was more pronounced for $ACTH^{1-24}$ than for the complete molecule $hACTH^{1-39}$ or for $hACTH^{1-32}$. This finding is in agreement with the hypothesis that the COOH-terminal part of ACTH protects the molecule to a certain degree especially against enzymatic degradation and at the same time inhibits the activity of the peptide itself by interaction with the receptor. Our study suggests that it is highly probable that these characteristics are caused by the sequence 25-32 of the molecule.
In addition to the well-known bioactive center of the molecule, the sequence 4-10, another centre in the peptide, within the sequence 11-19, has been found to induce corticosteroidogenesis as well.

Using the synthetic $hACTH^{1-39}$ as the standard the lowest limit of detection appeared to be 0.85 ± 0.47 pg/ml and using $ACTH^{1-24}$ as the standard the lowest limit was 0.20 ± 0.10 pg/ml. The ED50 in the assay proved to be 18.0 ± 6.9 pg/ml for $hACTH^{1-39}$ and 2.3 ± 0.8 pg/ml for $ACTH^{1-24}$ (4.0 ± 1.5 fmol/ml for $hACTH^{1-39}$ and 0.8 ± 0.3 fmol/ml for $ACTH^{1-24}$). The precision of

the assay - expressed as the quotient of the standard deviation and the slope of the regression line of the standard curve - was 0.046 ± 0.016.

Chapter III describes the development of a sensitive radioimmunoassay for ACTH. The commercially obtained antibody was directed against the sequence 17-24. ACTH was iodinated by way of an enzymatic procedure using lactoperoxidase. The tracer was purified by extraction with glass powder and immediately before addition in the assay also by column chromatography with Sephadex G 50. The free hormone was separated from the antibody-bound hormone with the use of plasma coated charcoal. The sensitivity of the assay proved to be 8.8 ± 2.8 pg $hACTH^{1-39}$/tube.

Chapter IV deals with the evaluation of the determination of ACTH in blood of normal subjects with aid of the assays described in chapter II and III. A direct measurement of ACTH in plasma was impossible due to interfering substances present in the plasma. Therefore, an extraction procedure has been developed for a reliable separation of ACTH from the interfering plasma substances. Several glass powders were tested, of which Vycor proved to be the only extractant useful for the measurement of ACTH in the bioassay and the radioimmunoassay. Dilutions of plasma extracts showed a parallelism with the standard curves both in radioimmunoassay and in bioassay. The mean intra-assay coefficient of variation ranged - dependent on the concentration of ACTH in plasma - in the radioimmunoassay from 6.3 to 6.5% and in the bioassay from 7.2 to 10.9%. The mean inter-assay coefficients varied in the radioimmunoassay from 10.4 to 12.3% and in the bioassay from 10.5 to 14.9%. The lowest detectable concentration of ACTH in plasma in the bioassay amounted to about 3.5 pg/ml and in the radioimmunoassay to about 10 pg/ml. It has been found that various standards show different responses both in the radioimmunoassay and in the bioassay. This implies that the standard to be chosen would strongly influence the height of the ACTH values measured. Much more is this true for ratios I-ACTH over B-ACTH when in each of these assays different standards would be employed. For this and other reasons synthetic $hACTH^{1-39}$ was used as the standard in both the assays. Using this standard in 17 normal subjects at 900 h in the morning a mean I-ACTH concentration of 48.7 ± 29.4 pg/ml plasma was found, and a mean B-ACTH concentration of 15.1 ± 8.8 pg/ml plasma. The mean I/B ratio amounted to 3.28 ± 1.09. These values point to the fact that only a part of I-ACTH is bioactive. This may be due to circulating ACTH metabolites which are recognized by the antibody but have an only very low bioactivity. As a result a dichotomy between the I-ACTH and B-ACTH plasma values

was observed. In spite of such dichotomy, I-ACTH values showed a correlation with B-ACTH values which reached a high statistical significance, indicating the usefulness of measuring ACTH by radioimmunoassay which for practical considerations has been chosen by many laboratories already.

The results of the clinical studies are described in chapter V. It appeared that the mean basal plasma concentrations as well as the ranges of I-ACTH, B-ACTH and cortisol in the control group of normal subjects yielded about the same values as in a control group of patients without clinical or biochemical evidence of disturbances in the pituitary-adrenal gland axis. The group of patients with untreated pituitary dependent Cushing's disease had significantly higher mean plasma levels for I-ACTH, B-ACTH and cortisol than both control groups. However, most of these values showed a wide overlap with the control levels. As might be expected the patients who had been treated for Cushing's disease by bilateral adrenalectomy and were off substitution therapy for at least 24 hours, had higher mean plasma ACTH levels than patients with Cushing's disease before treatment; in the patients treated and on substitution plasma ACTH levels were not significantly different from those of untreated patients with Cushing's disease. In the patients with Nelson's syndrome and in the patients with Addison's disease without substitution for at least 24 hours the mean plasma ACTH level was higher than in both control groups as well as in the group of patients with untreated Cushing's disease. No significant difference in the I/B ratio was found between both control groups. Patients with Cushing's disease showed a slightly higher I/B ratio than the normal subjects. After bilateral adrenalectomy the I/B ratio was no longer different from both control groups. Remarkably, the I/B ratio was significantly lower in the patients with Addison's disease as compared to that in the patients with Cushing's disease, but not lower than in the control subjects. In all groups studied a tight correlation was found between I-ACTH and B-ACTH. As was mentioned above the plasma levels of I-ACTH, B-ACTH and cortisol in patients with Cushing's disease showed a wide overlap with control subjects. Measuring both ACTH and cortisol in the same plasma samples it appeared that as many as 60% of the patients with Cushing's disease still had "normal" hormone levels. This indicates that such measurement in blood, collected at 900 h only, is of limited value for the diagnosis of Cushing's disease. The diurnal variation of the plasma hormone levels was studied in samples which had been collected at time intervals of 4 hours during the day. In the control patient group a circadian rhythm could be detected for I-ACTH, B-ACTH and cortisol. At 900 h the values were at their highest after which time these hormones were found to decrease and to reach their lowest

level at midnight. No such variations could be found in the patients with Cushing's disease. The I/B ratio did not show a circadian rhythmicity neither in the control patient group, and nor in the patients with Cushing's disease. A comparison of all values brought out that the plasma cortisol level is most valuable for discriminating for the diagnosis Cushing's disease.
For reasons of convenience the variation of the hormone plasma levels were studied at 900 h and 1600 h as well. Although the mean plasma I-ACTH, B-ACTH and cortisol levels were significantly lower at 1600 h than at 900 h in both control groups, individual values were higher at 1600 h than at 900 h in some control subjects. In the patients with Cushing's disease the 1600 h values were not significantly different from the 900 h values: the number of lower values almost equalled the number of higher values. In the patients with Addison's disease all I-ACTH levels and 5 out of 6 B-ACTH levels were lower at 1600 h than at 900 h.
In none of the groups studied a significant difference in the I/B ratio could be detected between the 1600 h and 900 h values.

It appeared that the plasma I-ACTH, B-ACTH and cortisol levels in the control groups were significantly lower at 930 h than at 900 h. In the patients with Cushing's disease no such short-term variation was observed. In none of the groups studied a statistically significant change in I/B ratio was found in this time interval. The variations in the hormone levels found between 900 h and 1600 h were also found between 900 h and 930 h. The question arises whether this short-term variation is caused by the diurnal biological clock or by a repeated veinous puncture. If the former is true, investigation of diurnal rhythmicity of ACTH and cortisol could be made more convenient by blood sampling in the morning at half hour intervals. If this short-term variation is a reflection of the diurnal rhythmicity investigation of this circadian variation could be made more convenient for practical reasons.
Overnight single dose dexamethasone administration decreased ACTH levels to not detectable levels in 9 out of 10 control subjects, whereas all ACTH and cortisol levels were much lower than the day before. In the patients with Cushing's disease all I-ACTH and cortisol values were higher than in the control subjects.

In chapter VI the relation between I-ACTH and B-ACTH was studied in control patients during insulin-induced hypoglycaemia. As expected a rapid increase of ACTH occurred about 10 minutes after the hypoglycaemia. Maximal ACTH levels were detected at 30 and 45 minutes after insulin administration.

Thereafter the ACTH levels decreased, B-ACTH disappearing faster than I-ACTH. Remarkably, the I/B ratio decreased (1.98) at rising ACTH levels. After reaching the maximal ACTH value the I/B ratio rose significantly to a value (6.54) exceeding the pre-treatment value (3.35). This effect might be explained as follows: in conditions in which ACTH is released the proportion of B-ACTH to I-ACTH increases. However when ACTH is decreasing after activation by hypoglycaemia the proportion of I-ACTH increases largely. This increase of I-ACTH relative to B-ACTH can be explained by a lower half-life of B-ACTH as compared to that of I-ACTH.

The results in this thesis can be summarized as follows 1) determination of ACTH by radioimmunoassay gives higher ACTH values than determination by bioassay. 2) close correlation between the values obtained by both assays both under normal and pathological conditions makes ACTH determination by radioimmunoassay as valuable as determination by bioassay for clinical diagnostic purposes. 3) the specific value of biological determination only becomes evident in conditions in which ACTH release from the pituitary gland is stimulated; under these circumstances increases of cortisol levels in blood are always preceded by a transient increase of bioactive ACTH relative to immunoreactive ACTH.

SAMENVATTING

In dit proefschrift is de ontwikkeling van een biologische en een radioimmunologische meetmethode ter bepaling van ACTH concentraties in humaan bloed beschreven. Het onderzoek omvatte de vergelijking van concentraties van immunologisch ACTH met die van biologisch actief ACTH in bloed onder verschillende physiologische, pathologische en experimentele omstandigheden.

De omvangrijke literatuurgegevens met betrekking tot een aantal eigenschappen van ACTH en diens gereguleerde afgifte uit de hypophyse zijn op beknopte wijze behandeld in de inleiding (hoofdstuk I). Hierin wordt ook ingegaan op het doel van de studie en wordt tevens een verantwoording gegeven voor de keuze van de gebezigde biologische meetwijze.

In hoofdstuk II volgt een uitvoerige beschrijving en evaluatie van de bioassay van ACTH zoals deze ontwikkeld werd. Met behulp van collagenase werden cellen geïsoleerd uit bijnierweefsel en vervolgens gesuspendeerd in een buffer. Teneinde een zo gevoelig mogelijke meetmethode te verkrijgen is een aantal wijzigingen in de oorspronkelijke assay - zoals deze in de literatuur het meest beschreven is - aangebracht en vervolgens getoetst. De belangrijkste verbeteringen die hebben geleid tot winst in gevoeligheid betroffen de zuivering van de geïsoleerde bijniercellen - door passage door een 5% BSA laag - gevolgd door een pre-incubatie van deze cellen. De winst in gevoeligheid ten gevolge van deze combinatie van zuivering en pre-incubatie bleek niet voor alle ACTH-peptiden even groot. Na zuivering en pre-incubatie, waardoor waarschijnlijk eiwitsplitsende enzymen worden verwijderd, was de winst in gevoeligheid van de assay groter voor $ACTH^{1-24}$ dan voor het volledige hormoon $hACTH^{1-39}$ of voor $hACTH^{1-32}$. Dit is in overeenstemming met bestaande hypothesen die aan het COOH-terminale deel van ACTH zowel een beschermende functie toeschrijven met name tegen enzymatische afbraak als ook een remmende functie met betrekking tot de interactie van de betreffende peptiden met de receptor. Onze studie heeft aannemelijk gemaakt dat deze functies op rekening komen van de sequentie 25-32.
Wat betreft de inductie van de synthese van corticosteroiden werden aanwijzingen verkregen dat niet alleen de sequentie 4-10 maar ook de sequentie 11-19 hiertoe in staat is.

Bij de ontwikkeling van de biologische meetmethode werd, bij gebruik maken van synthetisch $hACTH^{1-39}$ als standaard, een onderste gevoeligheidsgrens

vastgesteld van 0,85 ± 0,47 pg/ml en, bij gebruik maken van $ACTH^{1-24}$ als standaard, van 0,20 ± 0,10 pg/ml. De ED50 in de bepaling bleek 18,0 ± 6,9 pg/ml te bedragen voor $hACTH^{1-39}$ en 2,3 ± 0,8 pg/ml voor $ACTH^{1-24}$. De precisie van de assay - op de gebruikelijke wijze uitgedrukt als het quotient van standaarddeviatie en helling van de regressielijn van de standaardcurve was 0,046 ± 0,016.

Hoofdstuk III beschrijft de ontwikkeling van een gevoelige radioimmunoassay voor ACTH. Hierbij werd gebruik gemaakt van een in de handel verkrijgbaar antilichaam dat gericht is tegen de aminozuur-sequentie 17-24. Voor het bereiden van met radioactief jodium gemerkt ACTH werd een enzymatische procedure toegepast gebruik makend van lactoperoxidase. Het aldus gejodeerde ACTH werd vervolgens gezuiverd door extractie met glaspoeder en vóór elke bepaling ook nog via kolomchromatografie met Sephadex G-50. Het vrije ACTH werd gescheiden van het aan antilichaam gebonden ACTH met behulp van met plasma behandelde koolstof. De gevoeligheid van de methode bleek 8,8 ± 2,8 pg $hACTH^{1-39}$ per buis te bedragen.

Hoofdstuk IV behandelt de problemen die bij het meten van ACTH in bloed van normale proefpersonen met behulp van in hoofdstuk II en III beschreven methoden aan het licht traden. Een directe meting van ACTH in plasma bleek niet mogelijk, gezien de storingen door factoren uit het plasma zelf. Daarom werd een extractie-methode ontwikkeld waarmee ACTH betrouwbaar kan worden gescheiden van storende bestandddelen in het plasma. Bij dit onderzoek werden een aantal soorten glaspoeders getest, waarvan als enige Vycor zowel in de bioassay als in de radioimmunoassay bevredigende resultaten te zien gaf. Verdunningen van plasma extracten gaven zowel in de biologische als in de radioimmunologische bepaling "parallelle" resultaten. De reproduceerbaarheid binnen één meetserie varieerde, afhankelijk van de concentratie ACTH in het plasma, voor de radioimmunoassay van 6,3 tot 6,5% en voor de bioassay van 7,2 tot 10,9%. Tussen de verschillende meetseries waren deze waarden voor de radioimmunoassay 10,4 en 12,3% en voor de bioassay 10,5 en 14,9%. De minimaal te detecteren hoeveelheid ACTH bleek bij de biologische meting ongeveer 3,5 pg/ml plasma en bij de radioimmunologische meting ongeveer 10 pg/ml plasma te bedragen.

Uit het onderzoek bleek dat de keuze van de standaard een grote invloed heeft op de verhouding van de gemeten hoeveelheid I-ACTH (immunoreactief ACTH tot B-ACTH (bioactief ACTH). Uitgaande van verschillende standaarden werden verschillende I-ACTH/B-ACTH (I/B) ratio's gevonden. Gebruik makend van synthe-

tisch $hACTH^{1-39}$ als standaard werden bij 17 normale proefpersonen te 900 uur 's morgens een gemiddelde I-ACTH concentratie gevonden van 48,7 ± 29,4 pg/ml plasma en een B-ACTH concentratie van 15,1 ± 8,8 pg/ml. De gemiddelde I/B ratio bedroeg 3,28 ± 1,09. De vermelde waarden suggereren dat slechts een deel van I-ACTH biologische activiteit bezit.

De resultaten van klinische studies zijn besproken in hoofdstuk V. Het bleek dat de gemiddelde basale plasma concentraties evenals de spreidingsbreedte voor I-ACTH, B-ACTH en cortisol bij normale proefpersonen te 900 uur 's morgens ongeveer dezelfde waarden bereikten als die bij een controle groep van patienten zonder stoornis in de hypophyse-bijnier as. De groep van patienten met een onbehandelde hypophyse-afhankelijke ziekte van Cushing hadden significant hogere gemiddelde waarden voor plasma concentraties van I-ACTH, B-ACTH en cortisol dan beide controle groepen. Niettemin was er geen scherpe scheiding tussen de waarden in beide groepen voor het merendeel van de gemeten spiegels. Volgens verwachting hadden patienten, die waren behandeld voor de ziekte van Cushing door middel van bilaterale bijnierextirpatie en die gedurende tenminste 24 uur niet gesubstitueerd waren met corticosteroiden, gemiddeld hogere plasma ACTH spiegels dan patienten met de ziekte van Cushing vóór de behandeling; bij de groep van behandelde patienten die wel corticosteroiden gebruikten bleken de ACTH concentraties niet te verschillen van die bij de groep van patienten met een onbehandelde ziekte van Cushing. De patienten met een syndroom van Nelson hadden ACTH spiegels die veel hoger lagen dan die van alle andere groepen. Patienten met de ziekte van Addison, zonder substitutie tijdens tenminste 24 uur, hadden gemiddeld hogere plasma ACTH spiegels dan de personen in de beide controle groepen en de patienten met de ziekte van Cushing die niet waren behandeld.

Tussen de beide controle groepen werd geen significant verschil gevonden in de I/B ratio. Patienten met de ziekte van Cushing hadden echter gemiddeld een hogere I/B ratio dan normale proefpersonen. Na bilaterale adrenalectomie was de I/B ratio niet verschillend van die van de controle groepen. Opvallend was dat de gemiddelde I/B ratio bij de patienten met de ziekte van Addison lager was dan die bij de patienten met de ziekte van Cushing maar niet lager dan in de controle groepen. In alle bestudeerde groepen bestond er een hoge correlatie tussen I-ACTH en B-ACTH waarden.

Boven is reeds vermeld dat de spiegels van I-ACTH, B-ACTH of cortisol bij een groot deel van patienten met de ziekte van Cushing niet verschillen van die in de controle groepen. Ook indien men zowel ACTH als cortisol in hetzelfde

bloedmonster meet blijkt dat ± 60% van de patienten met de ziekte van Cushing "normale" spiegels in bloed te hebben. Dit geeft aan dat dergelijke metingen in bloed dat rond 900 uur 's morgens werd verzameld van geringe waarde zijn voor de diagnose ziekte van Cushing.

De diurnale variaties in de hormoon spiegels werden bestudeerd in bloed dat in een etmaal om de 4 uur werd afgenomen. De controle personen toonden een dag/nacht rhythme voor I-ACTH, B-ACTH en cortisol. Bij deze proefopstelling werden om 900 uur 's morgens de hoogste waarden gevonden en te middernacht de laagste. Voor de groep van patienten met de ziekte van Cushing konden dergelijke rhythmen voor plasma I-ACTH, B-ACTH of cortisol niet worden aangetoond. De I/B ratio gaf noch voor de patienten met de ziekte van Cushing noch voor de controle groep een circadisch patroon te zien. Bij vergelijking van alle verkregen gegevens bleek dat de meting van cortisol in bloed afgenomen omstreeks middernacht het meest discriminerend is voor het stellen van de diagnose ziekte van Cushing.

Bestudering van de verandering in de hormonale bloedspiegels tussen 900 uur en 1600 uur gaf weliswaar een significant lager gemiddelde waarde in I-ACTH, B-ACTH en cortisol om 1600 uur te zien in beide controle groepen, individueel echter werden ook enkele hogere waarden gevonden. In de groep van patienten met de ziekte van Cushing waren de gemiddelde hormonale waarden om 1600 uur niet significant verschillend van de waarden om 900 uur 's morgens: ongeveer evenveel waarden om 1600 uur waren hoger dan de waarden om 900 uur als lager. In de groep van patienten met de ziekte van Addison waren alle I-ACTH plasma concentraties om 1600 uur lager dan die om 900 uur. Dit gold ook voor B-ACTH in 5 van de 6 onderzochte patienten.
In geen van de bestudeerde groepen werd een significant verschil gevonden tussen de ochtend- en middagwaarde van de I/B ratio.

Bij metingen van de hormoon spiegels in bloed 's morgens in een tijdsinterval van een half uur bleken de waarden gemeten om 930 uur in beide controle groepen significant lager dan die te 900 uur. Bij de ziekte van Cushing echter werd een dergelijke significante verandering niet waargenomen. In geen van de bestudeerde groepen veranderde de I/B ratio in dit korte tijdsinterval.

De veranderingen die werden waargenomen in de hormoon spiegels in bloed tussen 900 en 1600 uur kwamen kwalitatief overeen met die tussen 900 en 930 uur. Het is de vraag of deze laatste variatie ook op rekening van de diurnale biologische klok moet worden geschreven, of toch een gevolg is van het effect

van herhaalde vena-puncties. Het is wellicht van belang hiernaar onderzoek te verrichten. Indien immers de variatie ook als uiting van het diurnale rhythme beschouwd mag worden zou het onderzoek daarnaar in de praktijk sterk vereenvoudigd kunnen worden.

Een van de onderzoekingen die in de diagnostiek van aandoeningen van de hypophyse-bijnierschors-as vaak wordt gebruikt is de bestudering van het effect van een synthetisch corticosteroid (dexamethason) op de plasma spiegels van cortisol en ACTH. Bij alle normale proefpersonen waren de plasma waarden voor I-ACTH, B-ACTH en cortisol om 900 's morgens op de dag na inneming van dexamethason 's avonds te 2300 uur lager dan die op de morgen van de voorafgaande dag. Bij de patienten met de ziekte van Cushing waren alle waarden voor I-ACTH en cortisol hoger dan die van de controle groep.

In hoofdstuk VI werd de relatie tussen I-ACTH en B-ACTH bestudeerd bij controle patienten tijdens een door insuline geïnduceerde hypoglycaemie. Volgens verwachting trad een snelle stijging van ACTH op rond 10 minuten nadat de hypoglycaemie was opgetreden. Een maximum werd bereikt 30 tot 45 minuten na toediening van insuline. Vervolgens daalden de ACTH spiegels, de B-ACTH waarden overigens sneller dan de I-ACTH waarden. Het was opmerkelijk dat de I/B ratio bij stijgende ACTH spiegels daalde (1,98). Na het bereiken van de maximale ACTH waarde steeg de I/B ratio significant hoger (6,54) dan de uitgangswaarde in het begin van het onderzoek (3,45). In hoofdstuk VI wordt met het nodige voorbehoud uiteengezet dat dit verschijnsel als volgt geïnterpreteerd moet worden: in situaties waarin ACTH afgifte actief wordt gestimuleerd neemt de bijdrage van B-ACTH toe. Bij dalen van ACTH spiegels na activering door hypoglycaemie blijkt de bijdrage van I-ACTH t.o.v. B-ACTH sterk toe te nemen. De hierdoor optredende stijging van de I/B ratio kan voor een aanzienlijk deel worden verklaard door een lagere half-waarde tijd van B-ACTH dan I-ACTH zoals ook bij dit onderzoek gevonden is.

De resultaten in dit proefschrift kunnen als volgt samengevat worden:
1) met de radioimmunoassay worden duidelijk hogere ACTH waarden gemeten dan met de bioassay.
2) niettemin is voor klinisch-diagnostisch gebruik, meting door middel van immunoassay even bruikbaar gezien de hoge correlatie die er werd gevonden tussen de waarden verkregen met beide methoden zowel onder normale als pathologische omstandigheden.
3) pas uit waarnemingen in het laatste hoofdstuk wordt duidelijk welke eigen

merıtes de bıologısche ACTH metıng heeft: onder omstandıgheden waarbıj bıologısche prıkkels de afgıfte van ACTH door de hypophyse stımuleren blıjkt eerst dat de stıjgıngen van cortısol spıegels ın bloed altıjd werden voorafgegaan door een kortdurende relatıef sterkere toenemıng van bıologısch dan van ımmunologısch meetbaar ACTH.

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LIST OF FIGURES

Fig	I.1	The structure of ACTH	3
Fig	I.2	Composition the "ACTH family"	5
Fig	II.1	Purification of adrenal cells	15
Fig	II.2	Calibration curve of corticosterone	17
Fig	II.3	Measurement of corticosterone in cells and medium	18
Fig	II.4	The effect of shaking upon the ACTH-induced steroidogenesis	19
Fig	II.5	The effect of 7.65 mM calcium upon the steroidogenesis induced by low doses of ACTH	20
Fig	II.6	The effect of pre-incubation for 60 min upon the ACTH-induced steroidogenesis in purified cells	23
Fig	II.7	Effect of cell purification and pre-incubation on the sensitivity to $hACTH^{1-39}$ and $ACTH^{1-24}$ of adrenal cell suspensions with and without added adrenal homogenate	25
Fig	II.8	The effect of the incubation time upon the steroidogenesis induced by low doses of ACTH in pre-incubated, purified cells	27
Fig	II.9	The effect of different BSA batches on the sensitivity of the standard curve	30
Fig	II.10	The reproducibility in the bioassay as measured in two separate experiments for two different doses of $hACTH^{1-39}$	30
Fig	II.11	The effect of different doses of β-endorphin on the corticosteroidogenesis without and with $hACTH^{1-39}$ (12.5 pg)	32
Fig	II.12	Log-dose response curves for ACTH peptides which contain the $ACTH^{5-10}$ sequence	33
Fig	II.13	Log-dose response curves for ACTH peptides without the 5-10 amino acid sequence	35
Fig	II.14	The effect of different doses of $ACTH^{11-19}NH_2$, $ACTH^{11-24}$ and $ACTH^{7-13}NH_2$ upon the steroidogenesis induced by 25 pg $hACTH^{1-39}$	36
Fig	II.15	The effect of the replacement of lysine in position 11 upon the biological activity.	37
Fig	III.1	Chromatographic pattern of the tracer	48
Fig	III.2	The effect of the presence of plasma in the separation suspension on the B_o and the non-specific binding	50
Fig	III.3	The effect of the contact time with charcoal on the B_o and the non-specific binding	50
Fig	III.4	The effect of time after separation of the bound and free fractions after centrifugation on the B_o and the non-specific binding	51
Fig	III.5	Standard curve in eightfold	52
Fig	III.6	Crossreactivity of ACTH-derived peptides in the radioimmunoassay	52
Fig	III.7	Scatchard plot of a standard curve	53
Fig	IV.1	The extraction recovery of tracer ACTH, added to 4 ml ACTH-free plasma samples, using different quantities of different types of glass powder	60
Fig	IV.2	The influence of plasma extracts using different glass powders upon the standard curves in radioimmunoassay and bioassay	62
Fig	IV.3	The recovery of tracer and standard ACTH in different volumes of ACTH-free plasma as measured by radioactivity, radioimmunoassay and bioassay respectively	63
Fig	IV.4	The percentage of standard ACTH, added to 4 ml ACTH-free plasma, as measured by radioimmunoassay and bioassay	63

Fig IV.5 The percentage recovery of tracer ACTH in different plasma samples 64
Fig IV.6 The replicate inter-assay variation in a patients plasma pool 67
Fig IV.7 Parallelism of two types of processed standard curves 68
Fig V.1 The immunoreactive ACTH values at 900 h 81
Fig V.2 The bioactive ACTH levels at 900 h 84
Fig V.3 The I/B ratio at 900 h 86
Fig V.4 Plasma cortisol levels at 900 h 88
Fig V.5 The relation between I-ACTH and B-ACTH in the plasma of the normal subjects 89
Fig V.6 The relation between I-ACTH and B-ACTH in the plasma of the patient control group 89
Fig V.7 Relation between I-ACTH and B-ACTH in the plasma of patients with untreated Cushing's disease. 90
Fig V.8 Relation between I-ACTH and B-ACTH in the plasma of patients after bilateral adrenalectomy for Cushing's disease with and without substitution therapy 90
Fig V.9 Relation between I-ACTH and B-ACTH in the plasma of patients with Addison's disease 90
Fig V.10 The relation between I-ACTH and cortisol in plasma of patients with Cushing's disease 92
Fig V.11 The relation between B-ACTH and cortisol in plasma of patients with Cushing's disease 92
Fig V.12 Circadian rhythmicity of I-ACTH, B-ACTH and cortisol in plasma of control patients 93
Fig V.13 The I/B ratio during a 24 hours cycle in control patients and in patients with Cushing's disease 94
Fig V.14 Individual levels of I-ACTH, B-ACTH and cortisol in plasma of patients with Cushing's disease during a 24 hours cycle 95
Fig V.15 Circadian variation in patients with Addison's disease 97
Fig V.16 Variations in plasma I-ACTH, B-ACTH and cortisol between 900 h and 1600 h 98
Fig V.17 Relative variation of I-ACTH in plasma between 900 h and 1600 h 99
Fig V.18 Relative variation of B-ACTH in plasma between 900 h and 1600 h 100
Fig V.19 Relative variation of cortisol in plasma between 900 h and 1600 h 101
Fig V.20 The relations of I-ACTH and B-ACTH with cortisol in plasma of patients with Cushing's disease 102
Fig V.21 Short-term variation of I-ACTH in human plasma between 900 h and 930 h 103
Fig V.22 Short-term variation of B-ACTH in human plasma between 900 h and 930 h 104
Fig V.23 Short-term variation of cortisol in human plasma between 900 h and 930 h 105
Fig V.24 I-ACTH plasma levels after dexamethasone suppression 107
Fig V.25 B-ACTH plasma levels after dexamethasone suppression 108
Fig V.26 Cortisol plasma levels after dexamethasone suppression 109
Fig VI.1 Individual and mean response of plasma glucose, I-ACTH, B-ACTH and cortisol and of the I/B ratio in control subjects upon insulin administration 113
Fig VI.2 The effect of injection of physiological saline upon the individual and mean response of plasma I-ACTH, B-ACTH and cortisol and of the I/B ratio in control subjects 116

LIST OF TABLES

Table	I.1	ACTH sequence of mammalian species	2
Table	I.2	Historical survey of bioassays of ACTH	7
Table	II.1	Influence of the sedimentation time on the responsiveness of adrenal cells	21
Table	II.2	Effect of purification and pre-incubation on the ED50 of ACTH and on the NADPH regulated steroidogenesis	22
Table	II.3	Effect of a 30 min incubation of the medium of adrenal cells on the steroidogenic activity of $hACTH^{1-39}$	24
Table	II.4	Relative potencies of different ACTH peptides assayed by corticosterone production	27
Table	II.5	Effect of hypophysectomy on the ED50 in crude and purified, pre-incubated adrenal cell suspensions	28
Table	II.6	Specificity of the bioassay	31
Table	II.7	Potencies of ACTH derived peptides	34
Table	II.8	Effect of low doses of ACTH on corticosterone production (ng/tube) in purified, pre-incubated suspensions	38
Table	II.9	Precision of the bioassay	39
Table	II.10	Historical survey of adrenocortical cell suspensions	40
Table	II.11	Comparison of isolated adrenal cells assays with $ACTH^{1-24}$ as standard	42
Table	IV.1	Comparison of the effect of different materials and their activation at 700° C upon tracer extraction from 4 ml ACTH-free plasma as performed at 4° C	60
Table	IV.2	The effect of temperature during extraction upon the recovery of $hACTH^{1-39}$ in ACTH-free plasma as measured by bioassay	61
Table	IV.3	Recovery of $hACTH^{1-39}$ in 12 different patient plasma samples as compared with the recovery in ACTH-free plasma in the same experiments	65
Table	IV.4	Reproducibility of the assays	67
Table	IV.5	Parallelism in radioimmunoassay and bioassay with diluted extracts of different volumes of plasma of different patients	69
Table	IV.6	Comparison of 18 bioassays for the determination of plasma ACTH	70
Table	IV.7	Comparison of 21 radioimmunoassay techniques for the determination of plasma ACTH	71
Table	IV.8	Quantities of standard preparations as compared to the activity of 100 pg synthetic $hACTH^{1-39}$ in radioimmunoassay and bioassay	72
Table	IV.9	Plasma ACTH levels in 17 normal subjects at 900 h	73
Table	IV.10	I/B ratio's in 8 normal subjects at 4 times on the same day	76
Table	V.1	Correlation between I-ACTH or B-ACTH and cortisol at 900 h	91
Table	V.2	Percentages of plasma I-ACTH, B-ACTH and cortisol levels in Cushing's disease exceeding the normal range at different times	96
Table	V.3	I/B ratio's at 900 h and 1600 h	100
Table	V.4	Percentages and numbers of plasma samples with hormone levels at 1600 h below the 900 h levels	106
Table	V.5	Percentages and numbers of plasma samples with hormone levels at 930 h below the 900 h levels	106
Table	VI.1	Simultaneous plasma hormone profiles during insulin-induced hypoglycaemia in an example subject	114
Table	VI.2	Correlations of plasma I-ACTH, B-ACTH and cortisol during insulin-induced hypoglycaemia	115

NAWOORD

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De definitieve versie van het manuscript werd met veel geduld persklaar gemaakt door Joke Peren-van Anholt, nadat enkele wijzigingen in de Engelse taal met veel toewijding en liefde waren aangebracht door G Goverde-Lips.

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STELLINGEN

I

Meting van immunoreactief ACTH met een antilichaam dat het centrale deel van het ACTH molecule herkent resulteert in gemeten waarden die hoger zijn dan die verkregen met een meting die de steroidproducerende activiteit van ACTH als parameter neemt.

dit proefschrift

II

Voor diagnostische doeleinden voldoet een ACTH radioimmunoassay gebruik makend van een antilichaam dat het centrale deel van het ACTH molecule herkent in gelijke mate als een biologische meting.

dit proefschrift

III

Studies die betrekking hebben op de relatie tussen de structuur en de biologische activiteit van ACTH peptiden kunnen slechts tot een betrouwbaar resultaat leiden wanneer in het meetsysteem de afbraak van deze peptiden tot een minimum is teruggebracht.

dit proefschrift

IV

Binnen het ACTH molecule bevinden zich twee sequenties die in staat zijn de steroidgenese in de bijnierschors te induceren.

dit proefschrift

V

De metingen van "Big" ACTH in humaan plasma van normale proefpersonen zoals toegepast door Thoren et al dient met een "grote" korrel zout genomen te worden.

M Thoren, M Ajne & K Hall (1981) Acta Endocrinologica 96: 15

VI

Het voorkomen van ACTH in weefsels buiten de hypothalamus-hypophyse regio dient nauwkeurig geverifieerd te worden met behulp van een betrouwbare bioassay.

E S Orwoll & J W Kendall (1979) Clin Research 27 23A

L I Larsson (1978) Histochemistry 55. 225

VII

De titel "A sensitive radioimmunoassay for corticotropin using a fully biologically active ^{125}I-labeled ligand" wekt ten onrechte de indruk dat de gevoeligheid van de assay het gevolg is van het gebruik van de genoemde ligand.

D J Buckley, J Hagman & J Ramachandran (1981) Endocrinology 109: 10

VIII

Bij kwaliteitscontrole van steroidhormoon-receptorbepalingen dient aandacht te worden besteed aan de nauwkeurigheid waarmee de "efficiency" van de radioactiviteitsmeting is vastgesteld.

IX

Bij psychosomatische klachten zal raadpleging van een advocaat soms effectiever zijn dan raadpleging van een arts.

X

Het gebruik om in de laatste stelling bij een proefschrift blijk te geven van een overigens schijnbare aanwezigheid van humor bij de auteur dient afgeschaft te worden - zie stelling XV.

XI

Het "milieu exterieur" van een humaan embryo kan beschouwd worden als een phylogenetisch relict.

XII

Een consultatieve psychiatrische dienstverlening in een ziekenhuis draagt bij tot kostenbeperking in de gezondheidszorg.

S J Levitan & D S Kornfeld (1981) Amer J Psychiatry 138: 790

XIII

De suggestie als zou verzet tegen de technocratie zijn als "blaffen tegen de maan" dient in het kader van een massaal verzet tegen plaatsing van kernwapens herzien te worden.

Ph Mertens & H Koningsveld (1981) Wending september: 500

XIV

De in universitaire kring veel gebezigde termen "onderwijsbelasting" en "onderzoeksactiviteiten" geven een duidelijk inzicht in de mentaliteit die veel medewerkers met betrekking tot de aan hen toevertrouwde taken wensen ten toon te spreiden.

H J M Goverde

15 december 1981